UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Glycine receptor in rat cortical astrocytes: expression and function

Mestrado em Biologia Molecular e Genética

Tatiana Pinto Morais

Dissertação orientada por:
Doutora Cláudia Valente (FML-UL/IMM) e pelo Professor Doutor Rui Gomes (DBV-FCUL)

2015

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive

Ricardo Reis

Recomeça....
Se puderes
Sem angústia
E sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.

Miguel Torga

Index

Figure Index

Table Index

Acknowledgments

Este trabalho não é um ponto final, é apenas uma etapa numa jornada que está apenas no começo. Aqui ficam registados os agradecimentos àqueles que trilharam esta jornada ao meu lado.

Muito obrigada! Muito obrigada à minha orientadora, à Doutora Cláudia Valente, que tornou todo este processo possível, e que me guiou ao longo deste ano. Guiou-me sempre com uma palavra amiga, novas ideias e entusiasmo. Obrigada pelo cuidado, pela amizade, por todos os ensinamentos e pela partilha do fascínio pelas neurociências. Obrigada.

À Professora Ana Maria Sebastião gostaria de agradecer a oportunidade de trabalhar no seu laboratório, os conselhos e os ensinamentos.

Ao Professor Rui Gomes pela ajuda, enorme disponibilidade e prontidão na resolução de todos os assuntos legais que este trabalho acarretou.

À Doutora Sandra Vaz pela preciosíssima ajuda na resolução de todos os problemas relacionados com a técnica de Imagiologia de Cálcio, pelas discussões científicas e pela partilha de conhecimentos. Um obrigado é pouco para te agradecer.

À Rita Aroeira, André Santos e Filipa Ribeiro que estiveram sempre disponíveis a ajudar. Um agradecimento especial à Rita pela ajuda na manipulação dos astrócitos e pela enorme partilha de conhecimento sobre a sinapse glicinérgica.

A todos os colegas do laboratório que de alguma forma contribuíram para a realização deste trabalho. Em especial à Margarida, Catarina, Nádia, Rui, João, Cátia, Cláudia e Daniela que para além de colegas se tornaram amigos. Obrigada por todos os momentos partilhados ao longo deste ano, científicos ou não, que tanto me ensinaram.

Ao Pedro e à Haíssa por tornarem mais rápida a aprendizagem sobre o mundo dos astrócitos e por todo o cuidado e ajuda. O meu muito obrigado.

Aos meus grandes amigos de infância, à Andreia, Danilo, Sérgio, André, Filipe, Cristiano e Rafaela, por me ensinarem o valor da amizade. E aos meus LCSanos por todo o companheirismos e por viverem esta aventura comigo.

Aos meus companheiros de mestrado, Tiago, Catarina e Vanessa, obrigada por todo o companheirismo e entusiasmo ao longo destes 2 anos.

Cinco agradecimentos muito especiais, à Sara, à Carmo, à Catarina, à Rita e ao Mickael. À Sara por todo o seu companheirismo e amizade, por estar sempre pronta a ouvir e a partilhar. Não tenho palavras para te agradecer. À Carmo por estar sempre pronta para

ensinar e para aprender, por nunca se esquecer do valor da amizade. Catarina e Rita, estes anos não teriam sido os mesmos sem vocês. Ao Mickael por tudo o que me ensina, por ser único.

À minha família. Aos meus avós, tios, primos, e aqueles que não sendo família se tornam numa, à Bela e à tia Carmo. Um enorme obrigado pelo vosso apoio e amor incondicional. Um obrigado especial à Mariana, à tia Nela, à tia Celeste, à madrinha, à Joaninha e ao Bruno.

À minha prima Rita que me permitiu construir um lar a 300 km de distância de casa.

À Mel, por toda a Cãopanhia.

Ao meu irmão, por estar sempre presente, no melhor e no pior. Por ser tão diferente de mim, e mesmo assim ser tão igual.

Por fim, o maior agradecimento de todos, aos meus pais. Ao meu pai pelo seu amor e por desde cedo me ter ensinado que tenho de tentar ser a melhor, a melhor versão de mim mesma. À minha mãe por todo o amor, cuidado e partilha de conhecimentos, pela força e por me ensinar desde cedo a expandir horizontes e a lutar.

Abbreviation List

ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
Ca^{2+}T	Calcium transients
cDNA	Complementary DNA
CI	Calcium imaging
Cl^-	Chloride ion
CNS	Central nervous system
CPA	Cyclopiazonic acid
CTL	Control
DIV	Days in vitro
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides thrisphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethykenediamine tetraacetic
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
fura-2AM	Fura-2 acetoxymethyl ester
Gab	Gabazine
GABA	Gamma-amino butyric acid

$GABA_AR$	GABA receptor type A
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCS	Glycine cleavage system
GFAP	Glial fibrillary acidic protein
GPCRs	G protein coupled receptors
Gly	Glycine
GlyR	Glycine receptor
GlyT1	Glycine transporter 1
GlyT2	Glycine transporter 2
ICC	Immunocytochemistry
ICW	Intracellular calcium wave
IF	Immunofluorescence
IHC	Immunohistochemistry
IP_3	Inositol 1,4,5 trisphosphate
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
Mus	Muscimol
Na^+	Sodium ion
NMDA	N-methyl-D-aspartate
NMDAR	N-Methyl-D-aspartate receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate bufferred saline solution
PCR	Polymerase chain reaction
PDL	Poly-D-lysine hydrobromide
PFA	Paraformaldehyde
PLC	Phospholipase C
PMSF	Phenylmethysulfonyl fluoride
PNS	Peripheral nervous system

PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time polymerase chain reaction
RIPA	Ristocetin induced platelet agglutination
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
Stry	Strychnine
TBS-T	Tris buffered saline Tween-20
TEMED	N,N,N’,N’- tetramethylethylenediamine
WB	Western Blotting

Abstract

In the brain, the inhibitory neurotransmission is mediated by GABA, while in the spinal cord and brainstem is mediated by glycine. Recent studies confirmed the presence of glycinergic transmission markers in the brain, like glycine receptor (GlyR) and transporters. However, GlyR expression in brain astrocytes was not yet described.

Astrocytes are now considered active elements in synaptic transmission, acting in a structure named tripartite synapse. They respond to synaptic activity and modulate neuronal response by gliotransmitters release. Such release is controlled by intracellular calcium waves (ICW), the form of astrocytic excitability. The ICW can be propagated to other astrocytes across "gap junctions", leading to a rise in calcium transients (Ca^{2+}T). ICW are the way of astrocytic communication and can occur spontaneously or in response to a stimulus, like ATP. Although GlyR activation has important known brain functions, its effect upon ICW has not been studied.

This project's objective is to explore GlyR expression and function in rat primary cultures of cortical astrocytes.

Western blot revealed GlyR and gephyrin expression, while immunofluorescence analysis showed GlyR in the cytoplasm and processes of astrocytes. qPCR further identified GlyR subunits α1, α2 and β within the time in culture.

GlyR activation effect upon ATP-induced Ca^{2+}T in astrocytes was evaluated using calcium imaging. Glycine, a GlyR agonist, caused a dose-dependent reduction in Ca^{2+}T, and this effect was abolished by strychnine, a GlyR antagonist. It was also shown that the decrease in Ca^{2+}T is due to an inhibition of calcium release from the endoplasmic reticulum, which is mediated by Cl^-. Manipulation of microtubules dynamics, which impairs GlyR anchorage at the cellular membrane, led to a loss of GlyR activation effect.

Overall, the results obtained propose an astrocytic GlyR activation-mediated inhibitory effect upon ATP induced Ca^{2+} transients, which requires GlyR anchorage at the plasma membrane.

Keys words: Glycine, Glycine Receptor, Inhibition, Calcium waves, Calcium transients

Resumo

A neurotransmissão, isto é, a forma de comunicação do sistema nervoso, pode ser inibitória ou excitatória. No sistema nervoso central a neurotransmissão inibitória pode ser mediada por ácido gama aminobutírico (GABA) ou por glicina, tendo estes dois neurotransmissores locais de actuação distintos. Tradicionalmente, o neurotransmissor GABA é descrito como o principal neurotransmissor a actuar no cérebro, enquanto a glicina exerce as suas funções na medula espinal e tronco cerebral. A transmissão excitatória, por sua vez, é da responsabilidade dos neurotransmissores glutamato e aspartato.

Porém, evidências recentes mostram a existência de sinapses glicinérgicas no cérebro. Foi já descrita a expressão do receptor de glicina em neurónios e dos transportadores de glicina, tipo 1 e tipo 2, em neurónios e astrócitos. Contudo, a expressão do receptor da glicina em astrócitos não se encontra ainda descrita.

Nos últimos 20 anos um novo conceito de sinapse emergiu. Este conceito, denominado "sinapse tripartida", considera os astrócitos como um elemento activo da transmissão sináptica, capaz de modular e participar na neurotransmissão, e não apenas como mero suporte aos neurónios. Segundo este modelo de sinapse, os astrócitos respondem à actividade neuronal pela libertação de gliotransmissores, isto é, moléculas activas capazes de modular os estímulos sinápticos. Hoje em dia diversos gliotransmissores já foram descritos, tais como o ATP, o glutamate e a D-serina. A libertação destes é controlada pela excitabilidade astrocitária, que se baseia na ocorrência de ondas intracelulares de cálcio, que podem ocorrer espontaneamente ou como resposta a um estímulo. Estas ondas propagam-se para outros astrócitos por "gap junctions", funcionando como uma forma de comunicação entre astrócitos.

O receptor da glicina é um receptor composto por cinco subunidades proteícas, formando um canal pentamérico permeável a cloro. As subunidades que o formam podem ser subunidades α ou β. Se o receptor for apenas formado por subunidades α, diz-se um receptor homomérico e é descrito como exercendo função no espaço extra sináptico. Por sua vez, quando formado por subunidades α e β (3α:2β ou 2α:3β) é um receptor heteromérico e pode ser encontrado no espaço sináptico. O ancoramento do receptor no espaço sináptico é feito pela proteína gefirina, que se liga à subunidade β. O ancoramento na membrana celular depende da migração do receptor do citoplasma até à membrana, sendo esta migração dependente da interacção entre a gefirina e os microtúbulos. Farmacologicamente, o receptor é activado por glicina, β-alanina e taurina, seus agonistas (nesta ordem de potência), enquanto a estriquinina é um potente antagonista selectivo.

Este receptor desempenha um importante papel fisiológico em várias zonas do sistema nervoso central, contudo o seu efeito sobre as ondas intracelulares de cálcio, e consequente aumento dos transientes de cálcio intracelulares no encéfalo, não foi anteriormente estudado.

Assim, o presente trabalho pretende estudar a função do receptor da glicina, na sinalização entre astrócitos, através da avaliação do seu efeito nas ondas de cálcio, induzidas por ATP, por imagiologia de cálcio em culturas primárias de astrócitos corticais de rato.
De forma a confirmar a expressão do receptor da glicina em astrócitos, cortes histológicos de cérebro de rato (12 μm de espessura), com 12 semanas de idade, foram utilizados num ensaio de imunofluorescência. Para a técnica de imunohistoquímica foi efectuada uma marcação dupla. Foram utilizados marcadores dos astrócitos (a proteína GFAP), do receptor de glicina (o anticorpo mAb4a, que identifica o receptor total) e da subunidade α2 do receptor da glicina. Através deste ensaio foi possível observar que no cérebro de rato ocorre a expressão do receptor da glicina em astrócitos, tanto na área do córtex como na área do hipocampo.

Após confirmação de que o receptor da glicina é fisiologicamente expresso em astrócitos cerebrais de rato, foram utilizadas culturas primárias de astrócitos de córtex para estudar a expressão do receptor ao longo do tempo em cultura, a sua localização celular e ainda para efectuar uma análise funcional do mesmo.

Por western blotting, observa-se que o receptor da glicina, bem como a gefirina, são expressos em culturas de astrócitos sem que ocorram alterações de expressão estatisticamente significativas ao longo do tempo em estudo. Por sua vez, a subunidade β do receptor da glicina apresenta um aumento do nível de expressão ao longo do tempo, sendo este aumento estatisticamente significativo do dia 10 para o dia 18 de cultura.

Relativamente aos níveis de expressão de mRNA das subunidades do receptor da glicina, o mRNA da subunidade α1 sofre uma diminuição de expressão ao longo do tempo em cultura, ocorrendo o inverso para a subunidade β. Por sua vez, o nível de expressão de mRNA da subunidade α2 diminui entre o dia 10 e 14 em cultura, ocorrendo posteriormente um aumento de expressão entre o dia 14 e 18.

No que respeita à localização celular, avaliada por ensaios de imunocitoquímica, foi encontrada marcação para o receptor, bem como para as suas subunidades α2 e β, no citosol e no espaço perinuclear, ao dia 10, 14 e 18 de cultura. Por sua vez, a gefirina foi detectada no espaço perinuclear e no núcleo.

Após a caracterização do receptor da glicina em astrócitos de córtex cerebral foi feita uma avaliação funcional do mesmo. Para este propósito avaliaram-se os efeitos da sua activação

por glicina na indução de transientes de cálcio, a forma de excitação astrocitária, através da técnica de imagiologia de cálcio.

A perfusão de astrócitos com glicina a 500 μM revelou uma diminuição nos transientes de cálcio intracelulares induzidos por ATP. Este efeito foi revertido quando estriquinina 0.8 μM, um antagonista selectivo do receptor da glicina, é adicionada ao sistema de perfusão, confirmando que o efeito da glicina observado é mediado pelo receptor da glicina.

Após observação do efeito inibitório exercido, por activação do receptor da glicina, na indução dos transientes de cálcio nos astrócitos, e sendo o receptor da glicina um canal iónico permeável a cloro, testou-se a hipótese deste efeito ser mediado pela entrada de cloro na célula. Para tal, os astrócitos foram perfundidos com um agonista, muscimol 3 μM, e um antagonista, gabazina 10 μM, do receptor GABA tipo A, um canal iónico permeável a cloro, tal como o receptor da glicina, e que já foi descrito em astrócitos. A perfusão com muscimol mostrou uma diminuição estatisticamente significativa nos transientes de cálcio, que é revertida na presença de gabazina, confirmando assim que o ião cloro é o responsável pela diminuição dos transientes de cálcio.

A proteína gefirina, ligada à subunidade β do receptor da glicina, é uma proteína citoplasmática responsável pelo recrutamento e ancoramento do receptor de glicina na membrana celular, onde o receptor é activado, através de um transporte dependente de microtúbulos. Com o objectivo de estudar se o ancoramento do receptor da glicina na membrana altera os efeitos celulares por si mediados, os astrócitos foram perfundidos com nocodazole, um fármaco que afecta a polimerização dos microtúbulos. A perfusão das células com nocodazole 1 μM e glicina 500 μM demonstrou uma perda do efeito inibitório, quando comparado com o caso em que só a glicina é perfundida, revelando assim a necessidade do receptor da glicina estar ancorado na membrana celular para que possa mediar um efeito inibitório sobre os transientes de cálcio induzidos por ATP.

Sabendo que após a estimulação por ATP ocorre libertação de cálcio do retículo endoplasmático, surgiu a questão de saber de que forma a activação do receptor de glicina interfere com esta libertação de cálcio da reserva intracelular. Para este fim, as células foram perfundidas com CPA 10 μM, um fármaco que previne a libertação de cálcio do retículo através de cálcio ATPases. Quando as células foram perfundidas com CPA ocorreu uma diminuição nos transientes de cálcio, mas a perfusão simultânea de glicina e CPA suprimiu quase totalmente os transientes de cálcio. Estes resultados revelam uma ligação entre a entrada de cloro para dentro da célula (via activação do receptor de glicina) e a diminuição dos transientes de cálcio, que se deve à inibição da libertação de cálcio do retículo.

Depois de confirmada, por ensaios de imagiologia de cálcio, a necessidade de recrutamento do receptor para a membrana celular para potencial activação, foram realizados ensaios de imunocitoquímica em que os astrócitos foram incubados por 10 e por 60 minutos, com glicina ou com glicina e nocodazole. Por imunocitoquímica observou-se que quando os astrócitos são incubados com glicina o receptor de glicina é efectivamente recrutado para a membrana celular, delimitando-a. Por sua vez, quando são incubados com glicina e nocodazole o receptor aparece disperso por todo o citoplasma, não sendo recrutado para a membrana celular.

No seu conjunto, os resultados obtidos sugerem que o receptor de glicina é expresso em astrócitos e medeia um efeito inibitório nos transientes de cálcio intracelulares induzidos por ATP quando activado por glicina.

Palavras-chave: Glicina, Receptor da glicina, Inibição, Ondas de cálcio, Transientes de cálcio

Nota: Esta dissertação não seguiu as normas do novo Acordo Ortográfico.

1 | Introduction

The nervous system is a highly complex entity, with millions of cells organized in synapses. Despite its enormous complexity, the system continues to be an enigma, with still much to know.

This system, can be divided in central nervous system (CNS) and peripheral nervous system (PNS), composed, respectively, by the brain and spinal cord, and the autonomic and somatic nervous system.[1,2] At the cellular level, neuronal and non-neuronal cells build up the nervous system.[1]

Neurons are vital for nervous system functionality. They communicate through the generation and transmission of electrochemical signals that result in the synaptic release, by exocytosis, of molecules called neurotransmitters. Synapses are highly complex structures where a large number of proteins control neurotransmitters release from the presynaptic membrane and its effects at the postsynaptic site, modulating and amplifying signals between cells.[1,3]

The non-neuronal cells are called glial cells, corresponding to around 90% of brain cells. The first neuroglia observation was made by Virchow, and in his words “this connective substance forms in the brain, in the spinal cord, and in the higher sensory nerves a sort of putty (neuroglia), in which the nervous elements are embedded” (Virchow, 1856).[4,5] This group of cells comprises astrocytes, oligodendrocytes and microglia in the CNS, and the Schwann cells in PNS.[1] Concerning glial function’s, Schwann cells and oligodendrocytes produce myelin, which involve axons, granting a fast communication among neurons by rapid spread of electrical impulses. Microglia are the immune cells of the CNS, which control brain infections and remove the inappropriate synaptic connections, by phagocytosis, guaranteeing the correct neuronal development and maturation. Astrocytes interact with neurons, intimately, and play a role in brain homeostasis by the release of numerous gliotransmitters, the glial neuroactive transmitters.[1, 6, 7]

1.1 | Astrocytes as Glial cells

The first exhaustive neuroglia investigation was performed by Camillo Golgi, and he described a homogeneous cell population with a star-shaped morphology, lately named astrocytes by Michael von Lenhossek.[5, 8, 9] Astrocytes are cells that extend numerous processes that wrap synapses and fine blood vessels.[6, 10, 11] These are non-electrically excitable cells with a negative resting membrane potential (determined by a transmembrane K^+ gradient), low input resistance, and extensive gap junctions between them.[8, 12, 13] There are two classes of astrocytes: 1) Protoplasmic astrocytes (type I), found in brain’s gray matter, with a stellate shape morphology and irregular contours and whose processes are

intimately associated with synapses and blood vessels; 2) Fibrillary (or fibrous) astrocytes (type II), found in brain's white matter, which present regular contours in the fiber-like processes and are associated with neuronal axons.[6, 10, 11] Both type of cells express an intermediate filament named glial fibrillary acidic protein (GFAP), classically used as a marker for astrocytes in the central nervous system.[4, 12]

1.2 | Tripartite Synapse

1.2.1 | Role of Astrocytes in the information flow

For a long time astrocytes have been considered the brain glue, simply supportive cells for neuronal functions, maintaining an optimal microenvironment. But, in the last two decades, new data about synapses has pointed astrocytes as the active third element of the "tripartite synapse" (Figure 1), recognizing their specific and important role in brain function.[7, 14]

The concept of tripartite synapse suggests that the synapse is formed by the pre and post-synaptic neurons plus the associated astrocyte that envelopes the synapse. In these synapses, astrocytes and neurons communicate in a bidirectional way, which means that astrocytes exchange information with neurons. Astrocytes have the capacity of respond to synaptic activity of different neurotransmitters and discriminate between the activity of different pathways that use the same neurotransmitter. In another hand, they regulate synaptic transmission by the release of gliotransmitters that influence neuronal excitability and synaptic transmission.[4, 7, 14] For this reason, nowadays, astrocytes are accepted as active synaptic function elements, involved in synaptic function. They integrate, process and collect synaptic information and control synaptic transmission and plasticity. Additionally, these cells are responsible for the release of energetic substrates, essential for metabolic sustain of nervous cells.

It is possible to say that brain function is regulated by a web of activity including neurons and glia, where astrocytes modulate neuronal excitability and synaptic transmission.[7,14-16] According to Araque, astrocytes are perfectly positioned to 'listen' and 'talk' to synapses.[7]

1.2.2 | Intracellular calcium waves

At synapse, astrocytes answer to neurotransmitters released by neurons generating an increase in their intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).[15] The response can be limited to one astrocytic process (cellular projection) or it can propagate, as an intracellular calcium wave (ICW), originating a rise in cellular calcium transients, to other astrocytic processes in contact with other cells types or astrocytes. The level of neuronal activity, that originates the ICW, regulates the extension of the response. Calcium transient's increase is a result of endoplasmic reticulum Ca^{2+} mobilization, leading to an elevation in the concentration of cytosolic Ca^{2+}. The ICW results from different neurotransmitter concentrations, concerning

a huge number of cells in different temporal and spatial scales to accomplish a higher brain integration level. [14-18] The ICW is then able to cause signaling molecules release, even at distant sites from the initial excitation zone, which are not undoubtedly active.[15] For this reason, the $[Ca^{2+}]_i$ signal is currently accepted as the way of cellular communication between astrocytes.[19] This signal, caused by neurotransmitters release in the synaptic cleft, plays a crucial role in the bidirectional communication at synapse, since it leads to gliotransmitters release by astrocytes and consequently to neuromodulation.[14, 16, 18, 19] We can say that astrocytes play an important role in the modulation of synaptic transmission since there is a mutual communication between neuronal activity and astrocyte excitability.[1, 7, 14] Astrocytic modulatory actions can be exercised on glial, neuronal and vascular cells.[18]

A wide diversity of gliotransmitters have been shown to be released by astrocytes, like glutamate, D-serine, ATP, GABA, tumor necrosis factor alpha (TNFα), prostaglandins, atrial natriuretic peptide (ANP), eicosanoids and brain-derived neurotrophic factor (BDNF).[12]

In culture, astrocytes have been shown to express receptors for a wide variety of neurotransmitters and, as a consequence, the application of neurotransmitters has long been known to induce robust ICWs, which can be propagated.[10]

In the synaptic cleft, astrocytic activation starts with neurotransmitter release from neurons that will activate astrocytic membrane receptors. Neurons release a wide variety of substances, such as ATP and glutamate, that activates G protein coupled receptors (GPCRs) in astrocytes, leading to activation of phospholipase C (PLC), with the associated production of IP_3 (inositol-1,4,5-trisphosphate) and the activation of IP_3 receptors in the endoplasmic reticulum (ER). This will result in a rise in the calcium levels in the cytoplasm by the release of Ca^{2+} stored at the ER. The rise in calcium level will open hemichannels and activates other mechanisms of gliotransmitter release Ca^{2+} dependent, like exocytosis.[10, 20, 21] The activation of this molecular cascade is able to generate a wide variety of oscillatory Ca^{2+} signals. Plus this molecular signaling, activation of ionotropic receptors permeant to Ca^{2+}, by synaptic activity, can also induce the $[Ca^{2+}]_i$ increase.[18]

1.3 | Glycinergic Synapse

1.3.1 | Neurotransmittion

Neurotransmission can be inhibitory or excitatory. The inhibitory neurotransmission in CNS is mediated by Gamma-Amino Butyric Acid (GABA) and glycine. GABA is considered the main inhibitory neurotransmitter in the brain, whereas glycine is traditionally described as the major inhibitory neurotransmitter in spinal cord and brainstem. Glutamate and aspartate are responsible for excitatory actions in the brain.[1,22]

Glycine is a non-essential amino acid, with a double role in CNS. It is an inhibitory neurotransmitter, acting upon glycine receptor (GlyR) chloride (Cl^-) channels, and is able to act as a co-agonist of glutamate at ionotropic N-Methyl-D-aspartate (NMDA) receptor (NMDAR), potentiating excitatory neurotransmission.[22-26] Glycine bind NMDARs with 100 times higher affinity than GlyRs, but under physiological conditions glycine binding sites of NMDARs are saturated.[25]

Recently, evidences point to the occurrence of glycinergic synapses in the brain. Glycinergic transmission related elements, like GlyR and glycine transporters (GlyT1 and GlyT2) were recently found in hippocampus and cortex.[27-30] But, although astrocytes are the primary source of hippocampal glycine[26], there are no evidences of GlyR expression in these cells.

1.3.2 | The glycinergic synapse physiology

In neurons, when released in the inhibitory glycinergic synaptic (Figure 1) cleft, glycine activates strychnine-sensitive post-synaptic GlyRs, which are densely packed in the postsynaptic membrane.[24, 25] As a result of the agonist binding, occurs the opening of GlyR anion channel, which results in an incursion of Cl^- ions in the post-synaptic cytoplasm. The resultant post-synaptic membrane hyperpolarization increases the threshold for neuronal firing resulting in the inhibition of the post-synaptic neuron.[3, 24-26, 28]

Glycinergic transmission can then be terminated by a rapid uptake of the neurotransmitter, mainly mediated by GlyTs[24,26], into pre-synaptic glycinergic nerve terminals and nearby glial cells, and by a regulation of glycine concentration in extracellular space.[25, 31] GlyR activation may, as a consequence, stimulate NMDARs and voltage-gated Ca^{2+} channels, resulting in an intracellular Ca^{2+} elevation.[26] By these mechanisms, GlyRs regulate neuronal development, as well as excitability, and synaptic plasticity.[26]

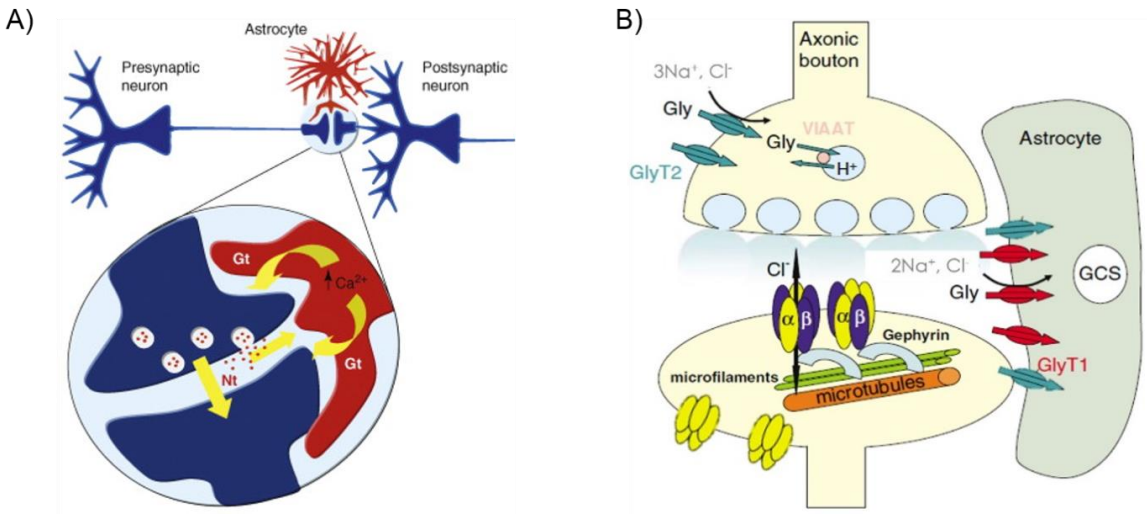


Figure 1: A tripartite synapse (A). The glycinergic synapse (B), adapted.[26, 32]

1.3.3 | Glycine Transporters

There are two GlyTs already described. Glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2), that can exist in several isoforms. They share around 50% homology in amino acid sequence but display different pharmacological functions. Glycine extracellular binding, jointly with Cl^- and Na^+, causes an alteration in the conformation of these transporters, causing a switch from an 'outward' to an 'inward' facing state.[24,28, 33]

It is accepted that GlyT1 is widely expressed in astrocytic cells, while GlyT2 is principally expressed in brainstem and spinal cord glycinergic terminals.[26,34] However, recently, the expression of GlyT2 in brain astrocytes has also been described.[24,28,29]

Glial GlyT1 has two main functions: 1) the glycine clearance from the inhibitory synaptic cleft, reducing the duration of the post-synaptic response; and 2) regulates excitatory neurotransmission at synapses containing NMDARs trough the control of glycine concentration.[24,26] Glycine transport via GlyT1 to astrocytes is made by a symport system through two Na^+ ions and one Cl^- ion. Inside the cell, glycine can suffer the action of the glycine cleavage system (GCS), being hydrolyzed by several enzymes.[35,36]

In turn, GlyT2 is responsible for the principal mechanism of glycine uptake at synapses, which is important for the restocking of neurotransmitter vesicles in presynaptic glycinergic neurons.[24,26,37,38] To execute the co-transported with glycine, GlyT2 needs three Na^+ ions and one Cl^- ion.[35] GlyT2 distribution mimics GlyR distribution, making this transporter and efficient marker for glycinergic nerve terminals.[28,39]

In summary, GlyT1 and GlyT2 have complementary functions: GlyT1 eliminates glycine from the synaptic cleft and, in that way, terminates glycinergic neurotransmission, whereas GlyT2 guarantees the restocking of vesicles in presynaptic glycinergic/mixed neurons.[24,26,37,38]

1.3.4 | Glycine Receptor

The ionotropic GlyR is the unique receptor for glycine known until now.[32] This receptor is a chloride channel that is part of the family of nicotinic acetylcholine receptor of ligand-gated ion channels, with none counterpart in the families of metabotropic receptors.[26,32] Intracellularly, GlyR can be found around the nucleus and in small aggregates dispersed in the cytoplasm.[40]

The receptor is composed by five protein subunits forming a pentameric channel that is permeable to chloride.[41] It can be composed by alpha (α) and beta (β) subunits, or only α, with, respectively, 48 and 58 kDa.[32] α subunits have high sequence identity (>80% homology) between them, but display significant sequence differences (<50% homology) if compared to β subunit.[42] Up until now four gene variants have been described to α subunits

(α1 - 4) and only one for β subunit.[32] Moreover, diversity in subunits can be achieved by alternative splicing.[43]

Each subunit is formed by proteins with four transmembrane domains (TM1 to 4). Regarding to protein insertion in the membrane, the amino and the carboxyl terminals are localized in the extracellular space. The amino terminal has a disulfide bond formed by four cysteine residues, giving the name to this family.[32] The connection between the transmembrane domain 1 and 2 is a small one, in turn, there are a large loop among TM3 and 4, which has important implications in synaptic GlyR anchoring and trafficking into and out of the membrane.[32,44]

Relatively to receptors design, functional homomeric receptors can be formed by only α subunits, while β subunit needs to be co-assembled with α subunits to form functional heteromeric GyRs.[32] In terms of arrangement, heteromeric GlyR can be composed by three α and two β subunits (3α:2β) or two α and three β subunits (2α:3β). This different composition can cause implications in GlyR function and pharmacology.[32, 45]

The α2 (49 kDa) subunit is the subunit with higher expression in immature spinal cord neurons, and homomeric extra-synaptic α2 receptors are ample during development.[29, 32,49] In turn, α1 (48 kDa) subunit is amply expressed in the mature spinal cord and brainstem neurons in association with β subunit. [29, 32,49] α3 subunit reflects the expression of the α1 subunit in mature neurons. The α4 subunit is a rare one.[32]

GlyR expression changes, over time, in rat hippocampus was recently explored. At birth, GlyR is composed by α2 and α3 subunits in a somatic localization and at low levels. Seven days after birth, there is an increase in receptor expression and some heteromeric α2β synaptic receptors can be found in the hippocampus. In more mature stages, occurs a decrease in α2β receptors expression, an increase in synaptic α1β and α3β and a progressive increase in extrasynaptic receptors containing α2 and α3 subunits.[29] This means that, in the brain, occurs a gradual replace of α2β by α1β receptors.[46]

The GlyR β subunit is broadly expressed in the nervous system, but its pattern of expression is different from the α subunit's pattern.[32,47] This subunit is responsible for the synaptic anchoring of GlyR by binding to gephyrin.[48-51]

Gephyrin is a key organizer for inhibitory post-synaptic receptors, essential for an efficient glycinergic signal transduction. It is a cytoplasmic tubulin-binding post-synaptic protein (93 kDa), composed by three main distinct domains, G, C and E.[40,44,52-54]

It forms oligomeric superstructures in the synaptic area, necessary for the postsynaptic clustering between the GlyR β subunit and intracellular microtubules, leading to an enlargement in the density of GlyRs in the postsynaptic membrane. The binding among the

two proteins is guaranteed by a hydrophobic interaction between gephyrin E domain and the cytoplasmic loop linking TM3 and 4 of the GlyR β subunit.[44,53-55]

One serine residue in the E domain controls the binding affinity to gephyrin, acting as a phosphorylation site.[52] Gephyrin has been detected in association with intracellular GlyR traveling throughout the cytoplasm, and the effect of nocodazole treatment, which interferes with the microtubule polymerization, points to a microtubule dependent transport.[40] Gephyrin can also be found bonded to $GABA_AR$ (GABA receptor type A) receptor, but this binding seems to be at least 10 times weaker than to GlyRs.[53] The GlyR-gephyrin interaction is reversible and very dynamic, being responsible for the regulation of GlyR diffusion and, as a consequence, for the GlyR density in the post-synaptic membrane.[26,40,56] Given this, in neurons, the α2 homomers are mostly extrasynaptic (activated by basal levels of glycine) while heteromeric receptors could be sequestered by gephyrin to a synaptic location.[32,57] Once in the synapse, gephyrin might work as a cellular sensor, adjusting inhibitory synaptic transmission in response to changes in activity.[52] Its functions are not only structural, gephyrin also regulates synaptic dynamics and interactions between proteins, making possible to have cytoskeletal proteins and downstream signaling proteins into close spatial proximity at the synapse.[52,53]

1.3.4.1 | Glycine Receptor Pharmacology

Glycine, β-alanine and taurine are glycine receptor agonists, in this order of potency, being the two latter regarded as partial agonists.[26,32,57] Concerning to inhibition, strychnine is a potent GlyR antagonist, selective and competitive towards glycine, which binds irreversibly to the α subunits. Picrotoxin, also used as a $GABA_AR$ receptor antagonist, inhibits glycine receptor activation by interfering, in an allosteric manner, with the glycine ion channel. Interestingly, picrotoxin appears to be able to distinguish between homomeric and heteromeric glycine receptors. β subunit is resistant to this drug, making the drug more selective for homomeric receptors.[58] For this fact, it is considered a useful indicator of the presence of heteromeric glycine receptors.[32,26,57]

2 | Aims

Despite recent evidences showing glycinergic synapses markers in brain cells, GlyR expression in astrocytes has not been proved, yet. For this reason, the main questions underlying this project are: “Does brain astrocytes express GlyR? If so, what is GlyR function?

To achieve this purpose, brain slices and primary cortical cultures of astrocytes were used to explore, at the molecular and functional levels, glycine receptor expression in brain astrocytes. Two specific topics were evaluated:

1. GlyR expression
   a) Assessment of GlyR expression in brain slices by IHC;
   b) Evaluation of GlyR expression, by Western Blotting and qPCR, and localization, by ICC, in primary cultures of astrocytes.
2. GlyR function, by calcium imaging experiments, in primary cultures of astrocytes
   a) Evaluation of GlyR activation effect on calcium transients induced by ATP;
   b) Assessment of intracellular mechanisms involved in the observed effect.

3 | Material and Methods

3.1 | Animals

This work used Sprague-Dawley rats, obtained from Charles River (Barcelona, Spain). All the procedures were performed respecting the European Union guidelines (2010/63/EY) and Portuguese law regarding the protection of animals for scientific purposes. The number of animals and their suffering were minimized.

3.2 | Primary cultures of astrocytes

Cultures enriched in astrocytes were prepared from the cerebral cortex of neonatal Sprague-Dawley rat pups (0–2 days), as described before.[28] Briefly, the animals were sacrificed by decapitation, followed by brain dissection in ice cold phosphate buffered saline solution (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$ and 1.5 mM KH_2PO_4, pH 7.4). After the removal of meninges and white matter the cerebral cortex was isolated. Cells were then vigorously dissociated in 4.5 g/l glucose Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Paisley, UK), supplemented with 10 % fetal bovine serum (FBS) (Gibco), 1 % antibiotic/antimycotic and glutamine. Cells were then filtered through a 70-µm cell strainer and centrifuged at 1200 rpm for 10 min at room temperature (RT). The pellet was resuspended in 4.5 g/l glucose DMEM, and filtered again through a 70-µm cell strainer (BD Falcon, NJ, USA) and centrifuged. The final pellet was resuspended in DMEM and then seeded according to the desired techniques.

Cultures were kept in an incubator with a humidified atmosphere (5% CO_2) at 37ºC and medium was changed twice a week. At 10 days in vitro (DIV) flasks were shaken for 5 hours in an orbital shaker at 300 rpm, in order to remove any contaminating microglia cells and thus obtain astrocytic-enriched cultures.[28]

3.3 | Immunofluorescence assays

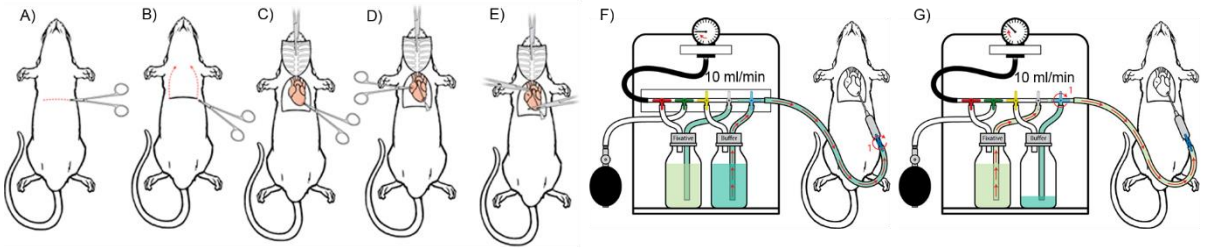
Detection of GlyR subunits, gephyrin and GFAP in rat cerebral slices and astrocytic primary cultures was performed by immunofluorescence assays.

3.3.1 | Immunohistochemistry

For immunohistochemistry studies, brains from 12 weeks old rats were used.

Slices' preparation: Briefly, at the day of the experiment, rats were deeply anesthetized with a mixture of Ketamina (120mg/kg) (Imalgene® 1000 Merial, France) and Xylazine (16mg/Kg) (Rompun® Bayer, Germany) by intraperitoneal injection, in a final volume of 0.1mL/0.1Kg of

body weight. The subsequent intracardiac perfusion was realized according to the following picture (Figure 2).



A - E) Thoracic cavity opening and heart revealing

F - G) Cannula introduction in the left ventricle. Perfusion with Buffer (0.9% NaCl) solution (F) folowed by perfusion with fixative (4%PFA) buffer (G).

Figure 2: Rodent intraperitoneal perfusion. Adapted. [59]

After perfusion, animals were decapitated, brains were removed and post-fixed by immersion in 4% PFA overnight at 4ºC. After a quick wash in PBS, brains were immersed in a 15% sucrose (in a 50 ml tube) solution at 4ºC. When the brains moved to the bottom of the tube, they were changed to a 30% sucrose solution. The tissue was embedded in gelatin and sliced (12 µm of thickness per slice), using a microtome, in the Laboratório de Histologia e Patologia Comparada of the Instituto de Medicina Molecular de Lisboa. Slices were stored at -20ºC until further use.

Antibodies staining: Slices were washed in PBS, at 37ºC for 10 min, in order to remove gelatin. Each slice was then surrounded with DAKO pen (Dako, Denmark), to protect staining areas from drying out and from mixing with each other, and washed with PBS. After 10 min of incubation in glycine 0.1M, which removes aldehydes left from the fixation step, slices were permeabilized for 10 min (0.1 % Triton X-100 in PBS). For GlyR detection, sections were subsequently immersed in fresh methanol, 10 min at -20ºC, and washed twice with PBS. After blocking for 3h, slices were incubated with the primary antibodies (Table 1: List of primary antibodies), diluted in the blocking solution, at 4ºC overnight, and with the fluorescent-labeled secondary antibodies (Table 2: List of secondary antibodies), also diluted in the blocking solution, for 90 min at RT. Nuclei were stained with Hoechst 33342 (1:100 dilution in PBS; Invitrogen) for 10 min at RT and the preparations were mounted in Mowiol (non-absorbing compound without autofluorescence and light scattering).

3.3.2 | Immunocytochemistry

For immunocytochemistry assays, astrocytic precursors were plated on poly-D-lysine hydrobromide (PDL) (25 µg/ml) coated 24-well plates and maintained for 18 days.

Cultured cells, at 10, 14 and 18 DIV, were fixed with 4% PFA in PBS for 15 min at RT, incubated 10 min in glycine 0.1M and permeabilized (0.1 % Triton X-100 in PBS) for 10 min.

The subsequent protocol was identical to the one performed in brain slices, with two small changes, the blocking and the secondary antibodies' incubation were carried out for 1h.

3.3.3 | Visualization

Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200, Germany) (Figure 3), using a monochrome digital camera (AxioCamMR3, Zeiss), with a 40x objective (Zeiss, Germany). AxioVision 4 software (Carl Zeiss Imaging Systems) was used for image acquisition. The obtained images were 1388x1040 pixels size, with an object space of 0.25µm/pixel.

Figure 3: Zeiss Axiovert 200. The microscope used for Immunofluorescence images acquisition.

3.4 | Western Blotting

Western blot assays were performed in order to study changes in the protein levels of GlyR and other related proteins.

Culture lysates: Cells were seeded into 60-mm dishes, and at day 10, 14 and 18 DIV cell lysates were obtained from the cultured astrocytes. Cell lysis was performed in 150 µL of RIPA (Ristocetin Induced Platelet Agglutination) buffer [50mM Tris pH 8.0, 1mM EDTA (Ethykenediamine Tetraacetic Acid), 150mM NaCl, 1% NP40 substitute (Nonyl phenoxlpoylethanol, from Fluka Biochemika, Switzerland), 1% SDS (Sodium Dodecyl Sulfate) and 10% glycerol]. To prevent protein degradation by endogenous proteases, RIPA buffer was supplemented with protease inhibitors (Complete Mini-EDTA free, Roche, Germany) and 1mM PMSF (phenylmethysulfonyl fluoride). The cell suspension was left shaking for 15 min at 4ºC and the insolubilized fraction was removed by centrifugation at 11000g for 10 min at 4ºC. Lastly, the supernatant was collected and stored at -20ºC for further use.

Protein Quantification: Total protein in lysates was quantified with Bio-Rad DC reagent (Hercules, CA, USA), using BSA (Bovine Serum Albumin) as the standard to establish the calibration curves.

Western blot assay: Samples were heated at 100 ºC for 10 min in order to denature higher order structures, while maintaining sulfide bridges. A 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the samples (40µg of protein per lane) and protein size marker (Precision Plus Protein Standards, Bio-Rad).[28] Subsequently, proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Millipore) at a constant voltage of 150V for 1h30, and blocked with 3% BSA in TBS-T (20 mM Tris base, 137 mM NaCl and 0, 1% Tween-20) at RT. Membranes were subsequently incubated with the primary (4ºC, overnight) and secondary antibody (RT, 1 h) (Table 1: List of primary antibodies and Table 2: List of secondary antibodies). Development of signal intensity was made by ECL Plus Western Blotting Detection System (Amersham-ECL Western Blotting Detection Reagents from GE Healthcare, Buckingamshire, UK) and visualized with the ChemiDoc™ XRS+Imager system (Hercules, CA, USA). The levels of relative expression of the protein bands were analyzed with Image J software and standardized for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Protein levels at 14 and 18 DIV were normalized to 10 DIV levels.

3.5 | Quantitative PCR (qPCR)

RNA isolation and quantification: Cells used in this assay were seeded into 60-mm dishes, as for western blotting. Total RNA was obtained from astrocytic cultures using QIAGEN RNeasy Mini Kit (Qiagen) and quantified with Nanodrop 1000 (ND-1000 Spectrophotometer, Thermo Scientific).

Reverse Transcription reaction: For the Reverse Transcription step, two reaction mixes were prepared, the RNA mix (3 µg of total RNA, 1 µL of random primers and 1 µL dNTPs, in a final volume of 10 µl) and the SuperScript mix [25 mM $MgCl_2$, 0.1M DTT (Dithiothreitol) and SuperScript II reverse transcriptase buffer, in a final volume of 10 µl].

The reverse transcription was executed in a thermoclycler (MyCycler – Bio-Rad, Hercules, CA 94547). RNA mix was heated for 5 min at 65 ºC and freeze for 2 min at 4ºC, followed by the addition of the SuperScript mix. 50 units of SuperScript II Reverse transcriptase (EC 2.7.7.49, Invitrogen, Carlsband, CA, USA) were added to the reaction when temperature reached 25ºC. Temperature was then raised to 42ºC (optimal SuperScript II temperature) for 60 min and the reaction was terminated by inactivating the enzyme for 20 min at 72 ºC.

Relative quantification: The cDNA amplification was operated in a Rotor-Gene 6000 real-time rotary analyzer thermocycler (Corbett Life Science, Hilden, Germany), using a SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.2 µM of each gene primer

(Table 3: qPCR primers). The amplification protocol was performed according to the next steps: denaturation for 2 min at 95ºC, 50 cycles of 30s at 94ºC, 90s at 60ºC and 60s at 72ºC, followed by a melting curve to evaluate the specificity of the reactions. The Rotor-gene 6000 Software 1.7 (Corbett, Life Science) was used to acquire the cycle Threshold (CT) and the melting curves (Appendix 2 | qPCR standard and melting curves). In order to perform a relative quantification by comparative Pfaffl method [60], a 5-fold sequential dilutions of cDNA sample was used to performed a qPCR for each pair of primers, with the aim of determine PCR efficiency (E) for each gene. Actin was used as the internal reference gene in all reactions. For each gene primer, duplication reactions were realized and the mean of the two reactions was used to calculate expression levels. Two types of negative controls were made, one reaction with cDNA obtained in the absence of SuperScript II and a second one without cDNA.

3.6 | Calcium Imaging

Calcium imaging experiments were performed to decipher GlyR function in astrocytes, using calcium transients as a function indicator.

For this assay, cells were plated on PDL (10 µg/ml) coated T75 flasks. At 10 DIV, after shaking, cells were replated in γ-irradiated glass bottom microwell dishes (MatTek Corporation, Ashland, MA, USA), coated with 10 µg/ml PDL.

Experimental design: Experiments used cells with 12 to 18 DIV. At the day of the experiment, cells were incubated for 45 min with the Ca^{2+} sensitive fluorescent dye fura-2 acetoxymethyl ester (fura-2AM; 5 µM; Calbiochem®, Darmstadt, Germany) at 22ºC. Cells were subsequently washed 3 times with a salt-rich solution (NaCl 125 mM, KCl 3 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 2mM, $MgSO_4$ 2 mM, D(+)-glucose 10 mM and HEPES 10 mM; pH 7.4 adjusted with NaOH) (Hepes buffer) and placed on an inverted microscope with epifluorescent optics (Axiovert 135TV, Zeiss, Germany) equipped with a xenon lamp and band-pass filters of 340 and 380 nm wavelengths. Throughout all experiments, cells were continuously perfused with the salt-rich solution (with or without added drugs) at 1.5 ml/second and visualized with a 40x oil-immersion objective.[61]

Cells were stimulated with 10 µM ATP for 200 ms by a FemtoJet microinjector (Eppendorf, Hamburg, Germany) through a pressure of 10 psi. In all experiments two stimulation trains were conducted. In the 1º train, which served as internal control, cells were stimulated with ATP at second 60, 240 and 420. After a fixed perfusion (1020s) in the drug-free Hepes buffer or with the experimental drugs, cells undertook the 2º train of ATP stimulation, at second 1440, 1620 and 1800, to assess the drugs' effect. Whenever a drug antagonist was used, the

perfusion of the antagonist started at second 240. The experimental design is represented in Figure 4.

The calcium transients amplitude, as response to ATP, was recorded by a ratiometric method, in which image pairs were obtained every 250 ms by exciting the preparations at 340 and 380 nm. Fura- 2AM has an absorbance of 340 nm if bounded to Ca^{2+}, and of 380 nm if not, but the emission wavelength is maintained at 510 nm. Excitation wavelengths were changed through a high speed wavelength switcher, Lambda DG-4 (Sutter Instrument, Novato, CA). The ratio between the emissions derived from the two excitation wavelengths (340/380) gives an estimation of intracellular Ca^{2+} concentration. All image data was recorded by a cooled CCD camera (Photometrics CoolSNAP) and processed and analyzed using the software MetaFluor (Universal Imaging, West Chester, PA, USA).[61] Regions of interest were obtained by delimiting the profile of the cells and averaging the fluorescence intensity inside the delimited area. The peak amplitude was calculated by subtracting the baseline level to the maximum peak intensity. The effect of each drug, evaluated in the 2º train of ATP stimulation, was calculated as a percentage of the response obtained in the 1º train.

The drugs and concentrations used in this approach are described in Table 4: List of drugs.

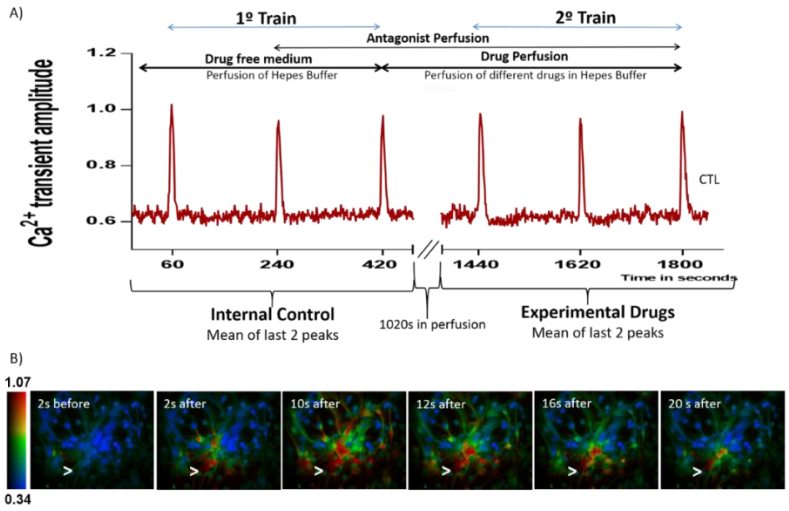


Figure 4: Scheme of the calcium imaging protocol. Representative plot of one control experiment (A). Ratio of fluorescence 340nm/380nm reflecting $[Ca^{2+}]_i$ before and after exposure to 10 μM ATP (B). Arrows represent the local of ATP pressure application.

3.7 | Statistical analysis

In this work, statistical significance was evaluated through the GraphPad Prism version 6 for Windows, GraphPad Software (San Diego California USA). Data are expressed as mean ± SEM from N independent cultures. In calcium imaging experiments the number of n responsive cells is indicated. One-way analysis of variance (ANOVA), followed by Bonferroni's Comparison Test, was used. Values of $p \leq 0.0001$ were considered to account for statistically significant differences.

4 | Results

4.1 | GlyR is expressed in rat brain astrocytes

Despite recent evidences of GlyR expression in rat brain, its expression in brain astrocytes has never been documented. In order to analyse GlyR expression in rat brain astrocytes an immunohistochemistry assay in adult rat brain slices was performed.

As described in section 3.3.1, adult rat brain slices (12 µm) were labelled with an antibody against GFAP, which served as a marker for astrocytes, together with mAb4a, which identifies GlyR, or the α2 subunit antibody. As demonstrated in Figure 5, GlyR is expressed in the cytoplasm and in the perinuclear space of astrocytes, in both cortex and hippocampus.

In both areas GlyR expression is higher than the α2 subunit expression, which indicates that astrocytic GlyR is not a homomeric α2 receptor. This assay show, for the first time, evidences of glycine receptor expression in brain astrocytes.

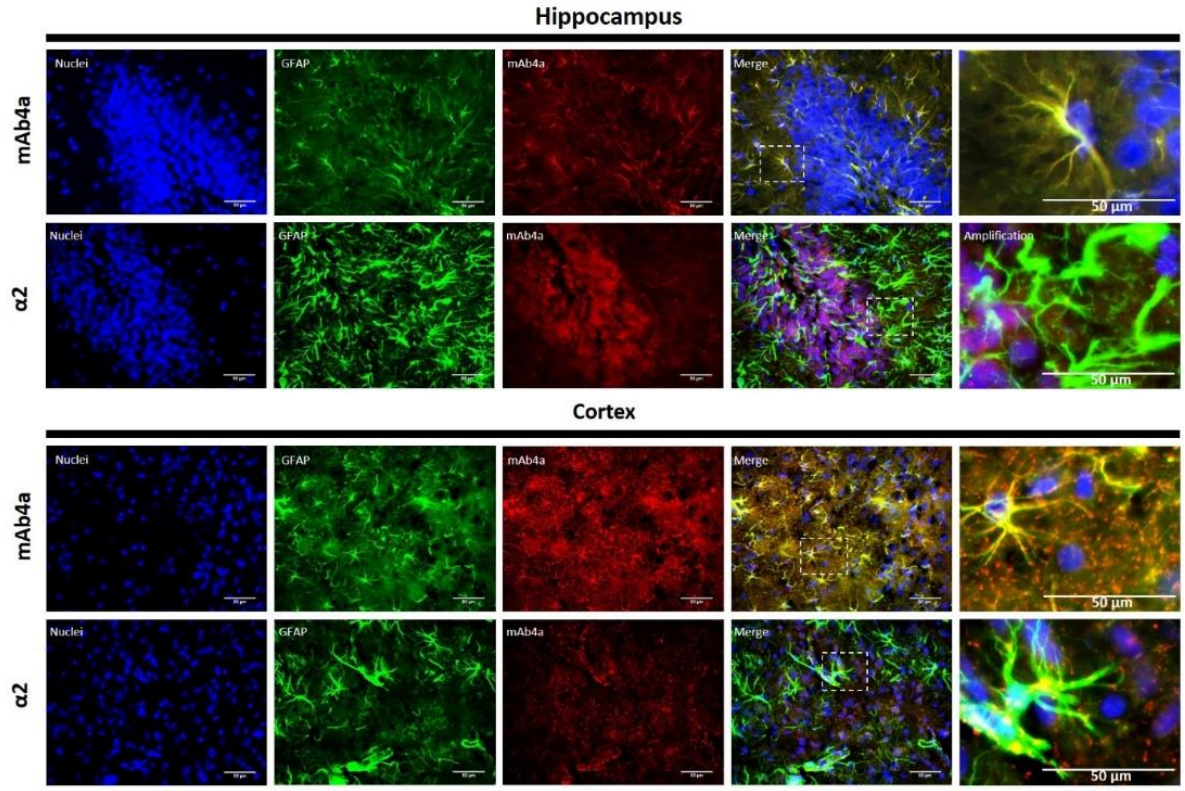


Figure 5: Double detection of GFAP and mAb4a/α2 subunit in rat brain slices. Nuclei were stained with Hoechst, GFAP stained astrocytes are green and mAb4a/α2 immunoreactivity is red. Immunofluorescence images were acquired with a 40x objective in a Zeiss Axiovert 200. Dotted lines represent the amplified areas. Scale bar of 50 µm.

4.2 | GlyR is expressed in cortical cultures of astrocytes

In order to characterize astrocytic GlyR, primary cultures of astrocytes were performed. These cultures are enriched in astrocytes (97% GFAP positive cells), being suitable for the study of astrocytes in an independent manner.[62] The preparation of primary cultures of astrocytes is relatively simple, allowing to study cell development and function.

Considering all the advantages, these cultures were used to study GlyR expression and function in astrocytes throughout time in culture, namely at 10, 14 and 18 DIV.

4.2.1 | GlyR and gephyrin protein expression

Characterization of GlyR protein levels was measured through a western blot assay, performed with protein extracts from primary cultures of astrocytes. In this assays, GlyR, GlyR β subunit, Gephyrin, and GAPDH expression levels were identified using specific antibodies. GAPDH served as the internal control. The expression levels were measured throughout time in culture, between day 10 and 18 *in vitro*.

In all time points, the antibodies used detected a single band, thus showing high specificity. A homogenate of cultured neurons was used as a control.

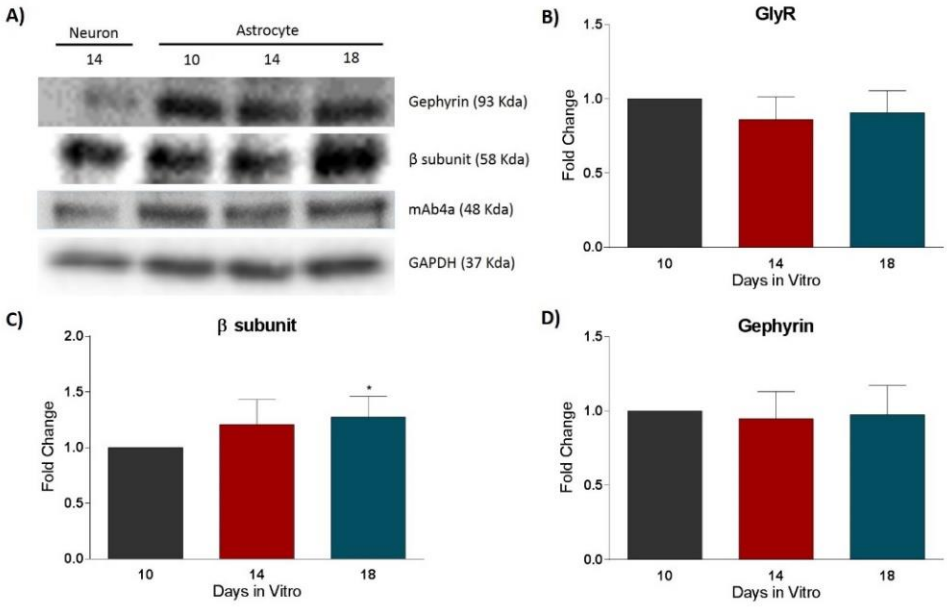


Figure 6: Analysis of GlyR expression in rat cortical astrocytic cultures by western blotting at 10, 14 and 18 DIV. Representative immunoblot (A) and densiometric analysis of mAb4a (B), GlyR β subunit (C) and Gephyrin (D) is shown. GAPDH was used as internal control. The densitometric analysis was performed with the ImageJ software. All values are mean ± SEM. N=3-8, *p≤0.05, one-way ANOVA followed by Bonferroni's Comparison Test.

The densitometry analysis (Figure 6 - B, C, D) shows that within time in culture there is a tendency for a decrease in GlyR expression, at 14 (0.86 ± 0.05806) and 18 (0.9071 ± 0.05571) DIV, compared to 10 DIV, but this change is not statistically significant. An opposite

tendency was observed for GlyR β subunit, where an increase in expression level occurred at 14 (1.208 ± 0.1003) and 18 (1.273 ± 0.1087) DIV, when compared to 10 DIV. However, only at 18 DIV this increase was found to be statistically significant (p≤0.05). On the other hand, gephyrin expression levels remained constant throughout time in culture (14 DIV: 0.9480 ± 0.08206 and 18 DIV: 0.9750 ± 0.09811).

The neuronal lysate was used to demonstrate that the antibody staining was accurate. As illustrated in the immunoblot (Figure 6 - A), all bands in the astrocytic lysates are similar to the ones obtained in the neuronal lysate.

These results unveil that, in culture, cortical astrocytes express components of the glycinergic synapses.

4.2.2 | mRNA expression of GlyR subunits

The mRNA expression of GlyR subunits in cultured astrocytes within time was achieved by real time PCR (RT-PCR) with specific oligonucleotide primers (Table 3: qPCR primers). All assays included a melting curve in order to assess primer specificity (Appendix 2 | qPCR standard and melting curves).

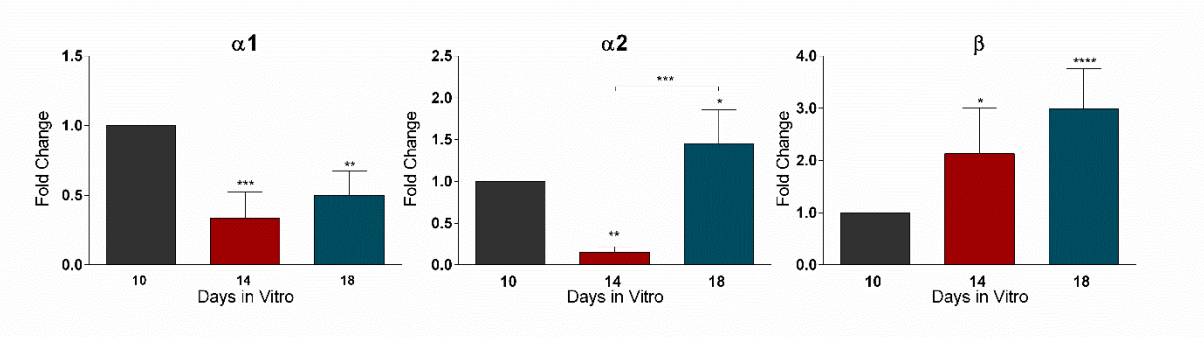


Figure 7: GlyR subunits mRNA levels, evaluated by qPCR, in rat cortical cultures at 10, 14 and 18 DIV. All values are mean ± SEM. N=3-8, * p≤0.05, ** p≤0.01 *** p≤0.001, **** p≤0.0001, one-way ANOVA followed by Bonferroni's Comparison Test, using 10 DIV as a control.

qPCR shows that mRNA expression of GlyR α1 subunit (Figure 7) undergoes a statistically significant decrease within time in culture, in relation to 10 DIV (14 DIV: 0.3350 ± 0.1909 and 18 DIV:0,5 ± 0.1732). In turn, GlyR α2 mRNA expression undertakes a decrease from 10 to 14 DIV (0.1550 ± 0.06364) and rises at 18 DIV (1.453 ± 0.4053). GlyR β subunit mRNA expression levels suffer a progressively statistically significant increase with time in culture, 14 DIV: 2.130 ± 0.8768 and 18 DIV: 2.997 ± 0.7579, in relation to 10 DIV.

4.2.3 | GlyR localization

The subcellular localization of GlyR, GlyR α2 and β subunits, as well as gephyrin, was investigated by immunocytochemistry at 10, 14 and 18 DIV astrocytes. A double staining of GFAP (astrocytic marker) together with GlyR, GlyR α2 subunit, GlyR β subunit or gephyrin was carried out. As in section 4.1, Hoechst was used as the nuclear marker.

In all time points studied, GlyR and its subunits, as well as gephyrin, were mostly distributed in the perinuclear space and in the cellular membrane. Gephyrin was also detected in the nuclei.

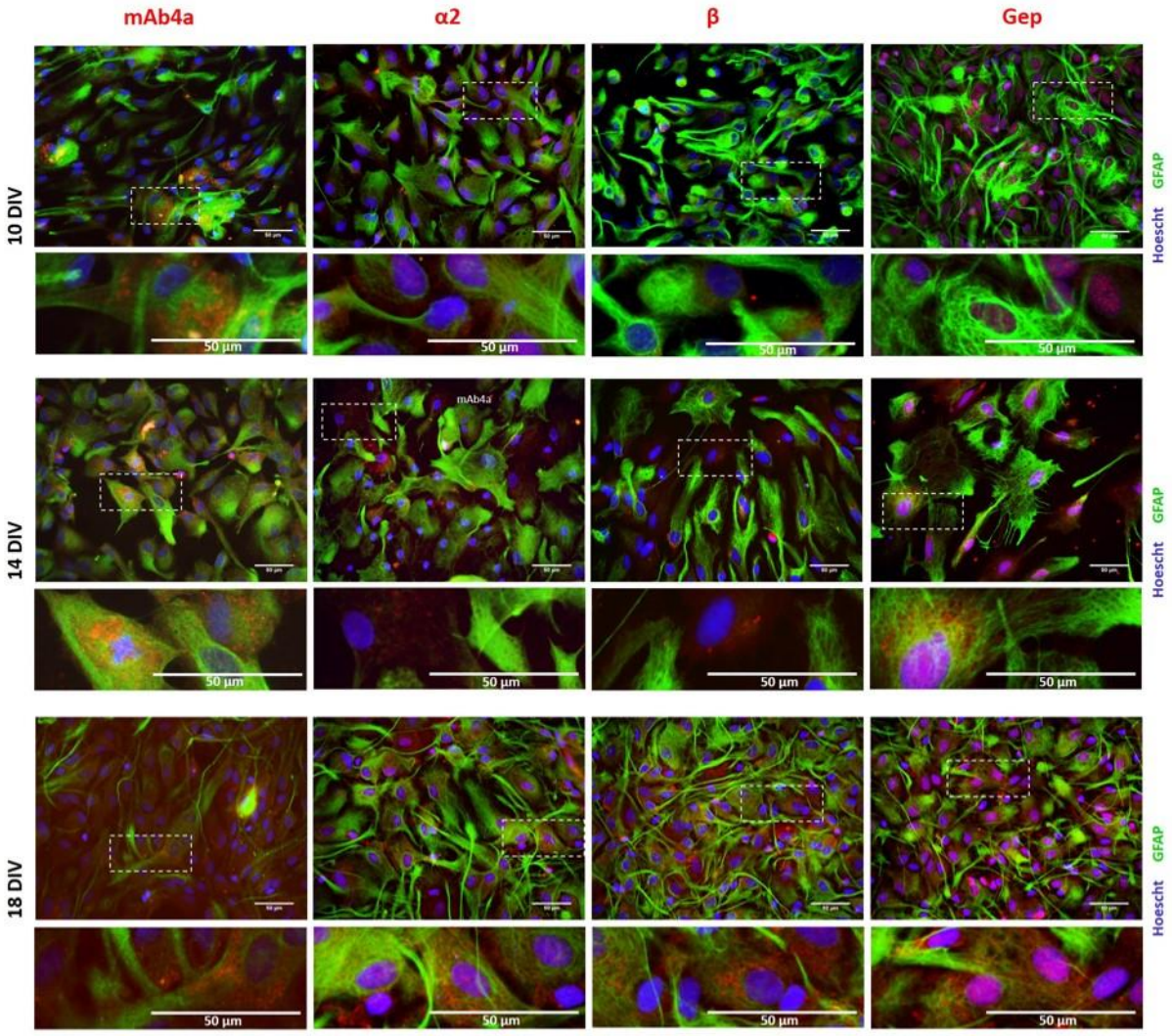


Figure 8: Double detection of GFAP and mAb4a/α2/β/Gephyrin in astrocytic cultures, at 10, 14 and 18 DIV. Nuclei were stained with Hoechst, GFAP stained astrocytes are green and mAb4a/α2/β/Gephyrin immunoreactivity is red. Fluorescence images were acquired with a 40x objective in a Zeiss Axiovert 200. Dotted lines represent the amplified areas. Scale bar of 50 µm. The single representation of each channel per picture is represented in the appendix (Appendix 3 | Fluorescence images, Figure 19).

4.3 | GlyR activation, by glycine, impairs Ca^{2+} transients in cortical cultures of astrocytes

4.3.1 | Glycine mediates a dose dependent inhibition in calcium transients

The purpose of the calcium imaging experiments was to accomplish a functional characterization of GlyR in cultured astrocytes, using calcium transients as an indicator of the performed functions.

In order to determine the best glycine concentration to be used in the functional assays, a dose response curve (Figure 9) was carried out.

In these assays, cells were stimulated according to the described methodology (3.6 | Calcium Imaging), and perfused with glycine concentrations from 10 µM to 10 mM. ATP stimulation (10µM for 200ms) causes a fast induction of calcium transients in cultured astrocytes, resulting in a peak representing the rise in cytosolic calcium, which briefly returns to a basal level. To exclude that the observed effects were derived from time (exhaustion or drug effects per si) or any other exterior factors, all experiments were done in the same conditions. Two separated trains of ATP stimulation were always performed. In the control situation (drug-free perfusion) the peak amplitudes were similar in the 1º and 2º trains. In turn, drug perfusion causes a decrease in the peak amplitudes of the 2º train, compared with the 1º (internal control). This decrease is not derived from protocol's design, since in the control situation astrocytes do not depict such decrease in calcium transients and thus, is associated to drug effect.

The concentrations used to perform the dose-response curve were chosen according to literature and physiologic concentrations of glycine in the nervous system.

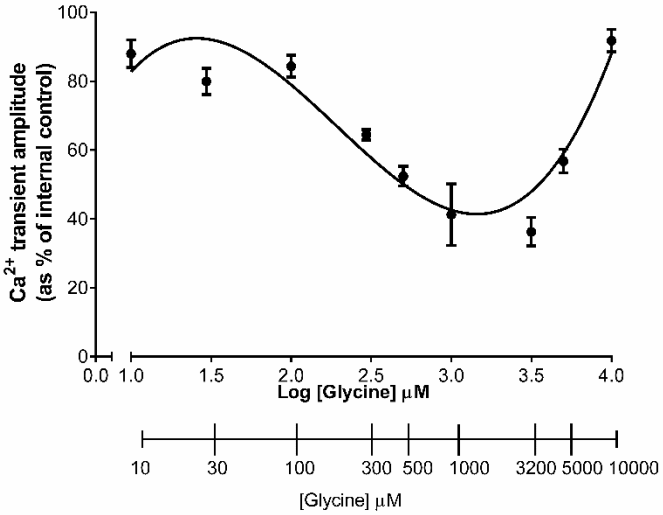


Figure 9: Glycine dose-response curve. Each point of the curve represents the mean of the cellular response when cells are perfused with glycine in a dose range between 10-10000 µM. The adjustment curves were obtained by a third order polynomial non-linear regression analysis. All values are mean ± SEM. N=2-3 culture plates.

By analysing the dose-response curve (Figure 9) it is possible to observe that glycine exerts a dose dependent inhibitory effect in ATP induced Ca^{2+} transients. This inhibitory effect increases with increasing glycine concentration and reaches a maximum around 3.2 mM of glycine. Above this glycine concentration the inhibitory effect is lost, probably due to GlyR internalization.

In order to analyse only the inhibitory effect of glycine, a non-linear regression of log (glycine concentration) vs. response was performed (Appendix 4 | Inhibitory dose - response curve, Figure 21), using the values of the inhibitory phase of the third order polynomial equation. The IC50, the concentration of the inhibitor that reduces the response by half, obtained from the curve as 430.9 µM.

Thus, the calcium imaging assays were performed with glycine 500 µM. Also, 500 µM of glycine was previously used in calcium imaging experiments, to study GlyR activation, by glycine, in oligodendrocytes progenitor cells.[63]

4.3.2 | Glycine activates GlyR and its effect is blocked by strychinine

To confirm that the observed glycine effect was mediated by GlyR activation a group of experiments was performed.

GlyR specific blockage allows to discard the participation of other receptors in the observed effect. This blockage was done with Strychnine (Stry), 0.8 µM, a drug which selectively blocks GlyR.

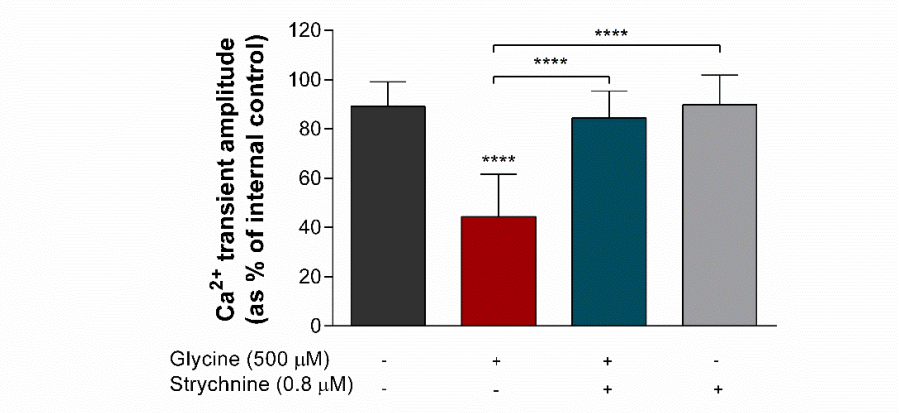


Figure 10: GlyR activation decreases ATP induced Ca^{2+} transients in cultured astrocytes. Summary plot of Ca^{2+} transients amplitude, as percentage of internal control, in each experiment. All values are mean ± SEM. n= 33-42 responsive cells from 3-5 independent cultures. **** p≤0.0001, one-way ANOVA followed by Bonferroni's Comparison Test. Representative curves of each experiment can be achieved in Appendix 5 | Calcium Imaging representative curves, Figure 22.

As can be observed in Figure 10, when cells were perfused with glycine at 500 µM there was a significant change in the Ca^{2+} transients amplitude (44.32% ± 3.029), when compared with the drug-free control (89.13% ± 1.668), which means that glycine exerts an inhibitory effect in the amplitude of calcium transients.

In turn, when GlyR was blocked with Strychnine 0.8 µM, and glycine 500 µM was perfused, there was no significant changes in Ca^{2+} transients' amplitude (84.38% ± 1.868) in relation to control. These results indicate that the glycine effect is mediated by GlyR, since it was completely reversed by its blockade. Strychnine 0.8 µM does not have any effect per si, 89.84% ± 1.853 reduction in Ca^{2+} transients amplitude vs 89.13% ± 1.668 in the control situation.

In summary this data shows that GlyR activation has an inhibitory effect upon ATP induced calcium transients in astrocytes.

4.3.3 | Calcium transients decrease is mediated by Cl^-

GlyR is a chloride permeable channel. Therefore, the participation of the chloride ion (Cl^-) in the described inhibitory effect was addressed.

Since $GABA_AR$ is also a Cl^- channel, highly studied in the CNS and present in astrocytes, a pharmacologic modulation of this receptor was performed. Muscimol and Gabazine, $GABA_AR$ agonist and antagonist, respectively, were used in these experiments.

One group of experiments in which glycine and muscimol were perfused simultaneously (muscimol perfusion starts at second 240 and glycine's at 420), leading to both GlyR and $GABA_AR$ activation, were analysed to investigate the relation between the two Cl^- channel receptors.

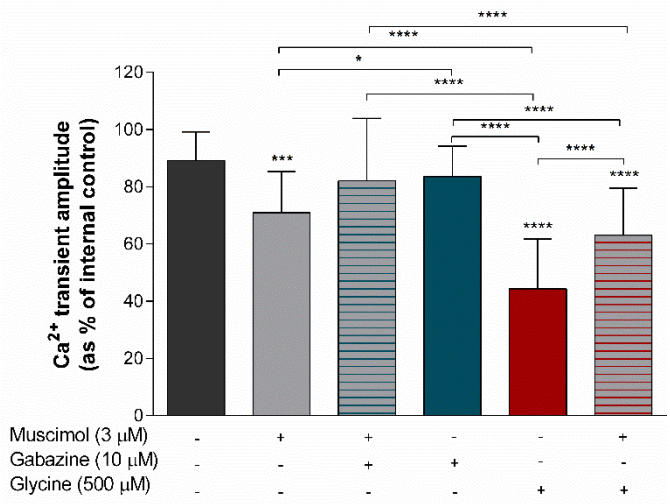


Figure 11: Cl^- mediates GlyR activation effect. Summary plot of Ca^{2+} transients amplitude, as percentage of internal control, in each experiment. All values are mean ± SEM. n= 25-49 responsive cells from 3-5 independent cultures; * p≤0.05, *** p≤0.001, **** p≤0.0001, one-way ANOVA followed by Bonferroni's Comparison Test. Representative curves of each experiment can be achieved in Appendix 5 | Calcium Imaging representative curves, Figure 23.

When muscimol (3 µM) was perfused (Figure 11) occurs a statistically significant decrease in the calcium transients (70.88% ± 2.896), compared to drug-free control (89.13% ± 1,668). But when Gabazine (10 µM) is added to the system, leading to the blockade of $GABA_AR$ and consequently to the inhibition of Cl^- passage through the channel, occurs a loss of the

muscimol inhibitory effect (Muscimol + Gabazine: 82.07% ± 3.983). Gabazine per si does not have any significant effect in calcium transients, 83.58% ± 1.516 decrease vs 89.13% ± 1.668 in the control situation.

In turn, when glycine and muscimol were perfused together the inhibition was higher (63.03% ± 3.004) than the observed when only muscimol was perfused (70.88% ± 2.896). Nevertheless, not as high as when only glycine was perfused alone (44.32% ± 3.029).

Altogether this dada indicates that Cl^-, passing through GlyR or $GABA_AR$ channels, mediates an inhibitory effect upon ATP induced calcium transients in astrocytes.

3.3.4 | GlyR anchorage is necessary for glycine effect upon Ca^{2+} transients

To better understand how GlyR acts in astrocytes, it's important to study GlyR anchoring at the cellular membrane. For this, the effect of nocodazole[64], an antimitotic agent that inhibits microtubule dynamics, was addressed (Figure 12).

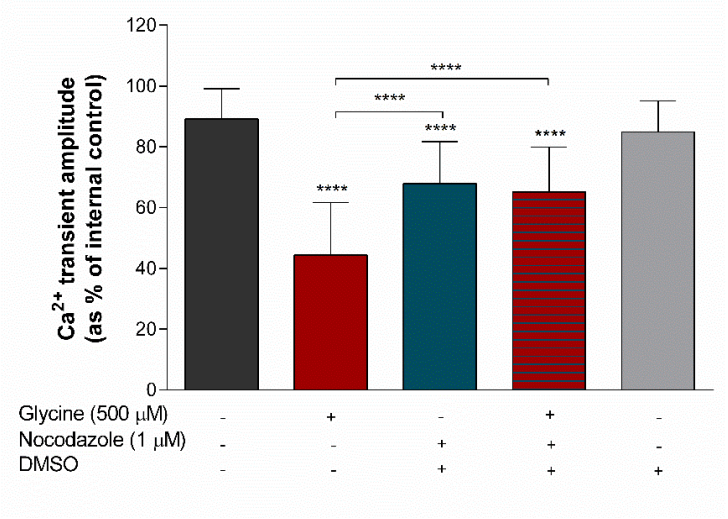


Figure 12: Nocodazole impairs GlyR activation effect upon ATP induced Ca^{2+} transients in cultured astrocytes. Summary plot of Ca^{2+} transients amplitude, as percentage of internal control, in each experiment. All values are mean ± SEM. n= 22-46 responsive cells from 3-5 independent cultures; **** p≤0.0001, one-way ANOVA followed by Bonferroni's Comparison Test. Representative curves of each experiment can be achieved in Appendix 5 | Calcium Imaging representative curves, Figure 24.

Cells' perfusion with nocodazole (1μM, diluted in DMSO 1%), caused a significant reduction in Ca^{2+} transients, compared to the drug-free control (67.95% ± 2.186 vs 89.13% ± 1.668), indicating that astrocytes are sensible to nocodazole treatment. DMSO, the nocodazole vehicle solution, compared to the drug free control does not have any significant effect (84.92% ± 2.174 vs 89.13% ± 1.668).

When glycine and nocodazole were perfused together no further reduction in calcium transients were obtained in relation to nocodazole alone, 65.17% ± 2.180 and 67.95% ± 2.186, respectively. This result indicates that microtubule dynamics' preservation is needed

for GlyR activation by glycine. In fact, microtubules are known to be essential for GlyR anchoring, through gephyrin, at the cellular membrane and without this anchorage GlyR cannot exert its actions.

4.3.5 | Glycine inhibits calcium release from the endoplasmic reticulum

To understand if the reduction in Ca^{2+} transients, caused by GlyR activation, is related with a decrease of Ca^{2+} release from the internal stores, or with Ca^{2+} exit from the cell, astrocytes were perfused with CPA. CPA is a specific endoplasmic reticulum Ca^{2+}-ATPases inhibitor[65], thus inhibiting the liberation of calcium from the principal cellular store to the cytoplasm.

As described by Jacob et al[61], CPA is related to the molecular cascade by which ATP, via P2Y receptor, acts in astrocytes. The endoplasmic reticulum Ca^{2+}-ATPases are the last component of this molecular cascade. Hence, CPA was used to examine if GlyR activation effect occurs through the endoplasmic reticulum, and, by this way, causes a decrease in free cytosolic calcium (Figure 13).

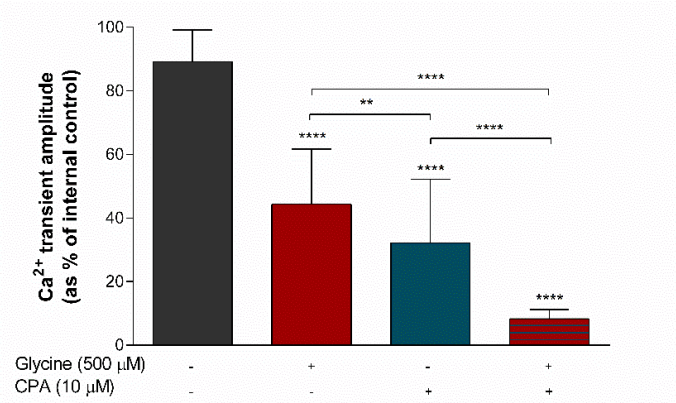


Figure 13: GlyR activation leads to a block of Ca^{2+} liberation from intracellular calcium stores in cultured astrocytes. Summary plot of Ca^{2+} transients amplitude, as percentage of internal control, in each experiment. All values are mean ± SEM. n= 33-44 responsive cells from 3-5 independent cultures; ** p≤0.01, **** p≤0.0001, one-way ANOVA followed by Bonferroni's Comparison Test. Representative curves of each experiment can be achieved in Appendix 5 | Calcium Imaging representative curves, Figure 25.

CPA (10 µM) perfusion leads to a marked reduction in Ca^{2+} transients (32.15% ± 3.015), compared to drug-free control (89.13% ± 1.668). In fact, higher than the one observed when glycine alone is perfused (44.32% ± 3.029). The combined perfusion of CPA and glycine leads to a more than 80% decrease in the Ca^{2+} transients amplitude (8.232% ± 0.4572), which means that the CPA effect is potentiated by GlyR activation.

Altogether this data indicates that GlyR activation, further inhibits calcium release from the endoplasmic reticulum, which, as a consequence leads to a decrease in Ca^{2+} transients amplitude.

4.4 | Glycine recruits GlyR to the plasma membrane

Knowing that nocodazole affects microtubules dimerization, which causes gephyrin loss of capacity to travel to the plasma membrane via microtubules partnership, it was expected that GlyR binding to the cellular membrane was compromised in the presence of nocodazole. Therefore, an immunofluorescence assay was performed to confirm the occurrence of changes in the cellular localization of GlyR in the presence of nocodazole, which would explain the loss of GlyR activation effect upon Ca^{2+} transients in the presence of nocodazole.

In order to disclosure if nocodazole treatment affects GlyR cellular localization, astrocytes from primary cortical cultures were incubated with glycine (500 µM) or with glycine and nocodazole (10 µM) for 10 or 60 minutes.

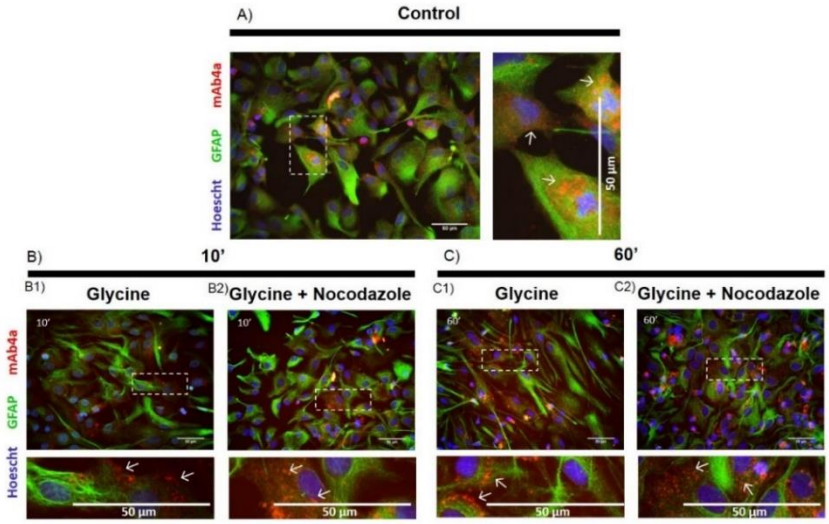


Figure 14: Double detection of GlyR and GFAP in 14 DIV astrocytes, in the presence of glycine and glycine + Nocodazole, for 10 or 60 min. Nuclei were stained with Hoechst, GFAP stained astrocytes are green and mAb4a immunoreactivity is red. Fluorescence images were acquired with a 40x objective in Zeiss Axiovert 200. Dotted lines represent the amplified areas. Arrows indicate GlyR localization. Scale bar of 50 µm. The single representation of each channel per picture is represented in Appendix 3 | Fluorescence images, Figure 20.

The results (Figure 14) indicate that, when astrocytes were incubated with glycine, a time dependent recruitment of GlyR to the cellular membrane occurs. In contrast, when cells were incubated with glycine and nocodazole, GlyR was confined to the cytoplasm and no delimitation of the cellular membrane could be observed.

5 | Discussion

Synapses are the functional units of the nervous system, where neurotransmitters coordinate brain functions, exchanging information. Regarding this fact, an exhaustive understanding of brain neurotransmitters and their receptors is imperative if one wants to achieve a holistic knowledge of brain function, essential for the development of new treatments and strategies to treat brain disorders.

Astrocytes have recently been pointed out as having an active role in synaptic transmission. This cells exchange information with synaptic elements and can modulate the received information at the synapses. Despite the recognized importance of astrocytes in synaptic transmission, the knowledge about these cells is still poor.

Glycinergic transmission was classically classified as a form of neurotransmission that takes place in the spinal cord and brainstem. However, recent research found glycinergic transmission markers in the brain. Functional glycine receptor in brain neurons, and glycine transporters in brain neurons and astrocytes were described, but glycine receptor expression in brain astrocytes was never explored. Glycine has been widely used to treat brain disorders, like schizophrenia.[66,67] In the last decade, glycinergic transmission as also been suggested to be a potential therapeutic target for epilepsy.[68,69] Derived from this, a vast knowledge about glycinergic transmission in the brain is imperious and will contribute for the discovery of new forms of treatment.

This work started with an evaluation of glycine receptor expression in rat cortical astrocytes.

Firstly, in order to unravel glycine receptor expression in physiologic conditions, glycine receptor expression was analyzed in rat brain slices. The results show, for the first time ever, that glycine receptor is expressed in cortical and hippocampal astrocytes.

Primary cultures of astrocytes were subsequently used to better evaluate receptor expression and cellular localization. No changes were found in protein expression levels within time in culture (between 10 and 18 DIV) for GlyR and Gephyrin, but GlyR β subunit expression upsurges in time, being the changes statistically significant between 10 and 18 DIV. Relatively to mRNA expression levels, the GlyR α1 subunit expression decreased throughout time in culture, while GlyR α2 demonstrated an initial decrease between day 10 and day 14 and an increase in the latest time point. Regarding GlyR β subunit, mRNA expression levels showed a statistically significant increase between 10 and 18 DIV. This increase in GlyR β subunit expression could be attributed to an increase in heteromeric GlyR with astrocytes' maturation. However, qPCR had some variability between cultures and thus these assays need further confirmation.

GlyR, mAb4a, α2 and β subunits were found in the plasma membrane and in the perinuclear space, while gephyrin was present not only in the cytoplasm, as expected, but also near the nuclei. The cellular localization of GlyR and GlyR α2 subunit are identical in brain slices and in cultured astrocytes and they are in accordance with studies performed in neurons.[40] The finding that gephyrin was present in the nucleus may be due to nonspecific staining. Glycine receptor was never studied in astrocytes, and, for that reason, this was the first description of its expression pattern and cellular localization in these type of cells.

The second aim of this work was to unravel GlyR function in astrocytes. To achieve this propose calcium imaging assays were performed to analyze the relationship between GlyR activation and astrocytic calcium transients.

The perfusion of astrocytes with glycine 500 μM was shown to diminish calcium transients, being the effect reversed by the addiction of strychnine at 0,8 μM. At the concentration used, strychnine is a selective GlyR antagonist, which binds GlyR in the binding site for glycine, indicating that the observed effect of glycine was due to GlyR activation.[63] In neurons and oligodendrocytes, a similar inhibitory response was observed by the application of glycine and strychnine, and thus a GlyR activation inhibitory effect was also reported.[26,32,63] The decreased concentration of intracellular calcium, compared to the control, after GlyR activation, could be related to a decrease in the liberation of calcium from the reticulum, described after ATP stimulation, or related to a crosstalk between Ca^{2+} permeable AMPARs and GlyR, which was already described in neurons.[26,61]

Since GlyR is an anion channel, permeable to Cl^-, the observation of the inhibitory effect of GlyR activation point to Cl^- as the mediator of this inhibition. $GABA_AR$, the GABA receptor type A, is also a Cl^- channel, alike GlyR, that is known to be expressed in astrocytes. For this reason, in order to disclosure if Cl^- was interfering with calcium transients, $GABA_AR$ was activated and the activation effect upon ATP induced calcium transients was evaluated. Muscimol, a $GABA_AR$ agonist, was able to induce a statistically significant decrease in calcium transients, which was reversed by $GABA_AR$ antagonist, Gabazine. Interestingly, GlyR activation exercises a more potent inhibitory effect on calcium transients than $GABA_AR$ activation, and when the two drugs were perfused together their effects were not cumulative. Studies in hippocampal neurons show a state-dependent cross-inhibition between these two receptors, GlyR activation can modulate $GABA_AR$, resulting in a depressed GABA-mediated response.[32,70,71] The opposite result has also been shown, with a GlyR-mediated depressed response under $GABA_AR$ activation.[32,70,71] In the present work only the effect of $GABA_AR$ activation in GlyR response was studied, and, as described in neurons, a cross-talk between the two inhibitory receptors was observed. In the future, is crucial to evaluate if, in astrocytes, the $GABA_AR$-mediated response is affected by GlyR activation. Furthermore, besides the

functional crosstalk between these two Cl^- channels, it would be interesting to address the occurrence of the crosstalk in astrocytes of a close proximity, or even a physical association between them.

Gephyrin is the protein responsible for GlyR anchoring in the plasma membrane, which recruits GlyR through microtubule transport. Astrocytes were perfused with nocodazole, a drug that affects microtubule polymerization,[64] in order to study if GlyR needs to be anchored at the plasma membrane to exert its inhibitory effect upon calcium transients. Nocodazole treatment caused the loss of GlyR inhibitory effect, proving the requirement of GlyR anchoring at the membrane in order to be activated and thus, exercise the inhibitory effect. In neurons, nocodazole treatment was shown to induce a decrease in the rate of GlyR accumulation at the cellular membrane and reduce the GlyR-Gephyrin small aggregates along the cytoplasm,[40,46] indicating that the stabilization of the receptor in the membrane was gephyrin dependent. Therefore, the findings shown here are in accordance with the ones reported in neurons.[40,46] Still, this was the first work to study, in astrocytes, the relation between microtubules and GlyR. The decrease in calcium transients in the presence of nocodazole could also be due to a loss of cellular microtubules, which may affect the cellular cascade induced by ATP activation of P2Y receptors,[61] but the joint perfusion of glycine and nocodazole did not show any statistical difference with nocodazole perfusion alone. These results further reinforce GlyR recruitment at the plasma membrane, as being the key element for GlyR-mediated inhibitory effect upon calcium transients.

The remaining question was how Cl^- inhibited calcium release from the endoplasmic reticulum. To answer this question, astrocytes were perfused with CPA, a drug that prevents Ca^{2+} release from the reticulum through an ATPase. When perfused with CPA, astrocytes showed a high decrease in calcium transients, as reported by others.[61] However, when glycine was perfused together with CPA, calcium transients were almost abolished, revealing a direct link between GlyR activation and calcium release from the endoplasmic reticulum. These results demonstrate a Cl^- role as an intracellular messenger in astrocytes.[72] So, in astrocytes, GlyR activation leads to an astrocytic inhibition because it inhibits calcium release from the endoplasmic reticulum.

The final question addressed in this work was related to GlyR cellular localization in the presence of glycine. It was hypothesized that glycine could be promoting the GlyR movement to the cellular membrane. To answer this question astrocytes were incubated with glycine 500 μM and glycine 500 μM + nocodazole 10 μM for 10 and 60 minutes. The results did show that, in the presence of glycine alone, GlyR moved to the membrane in a time dependent manner and, in the presence of glycine and nocodazole, this movement was completely impaired. Once again, these results point to the need of intact microtubules for

GlyR recruitment to the plasma membrane of astrocytes, which is in agreement with the reported in neurons.[40]

As a summary of the work, a model of GlyR activation in brain astrocytes is proposed in Figure 15.

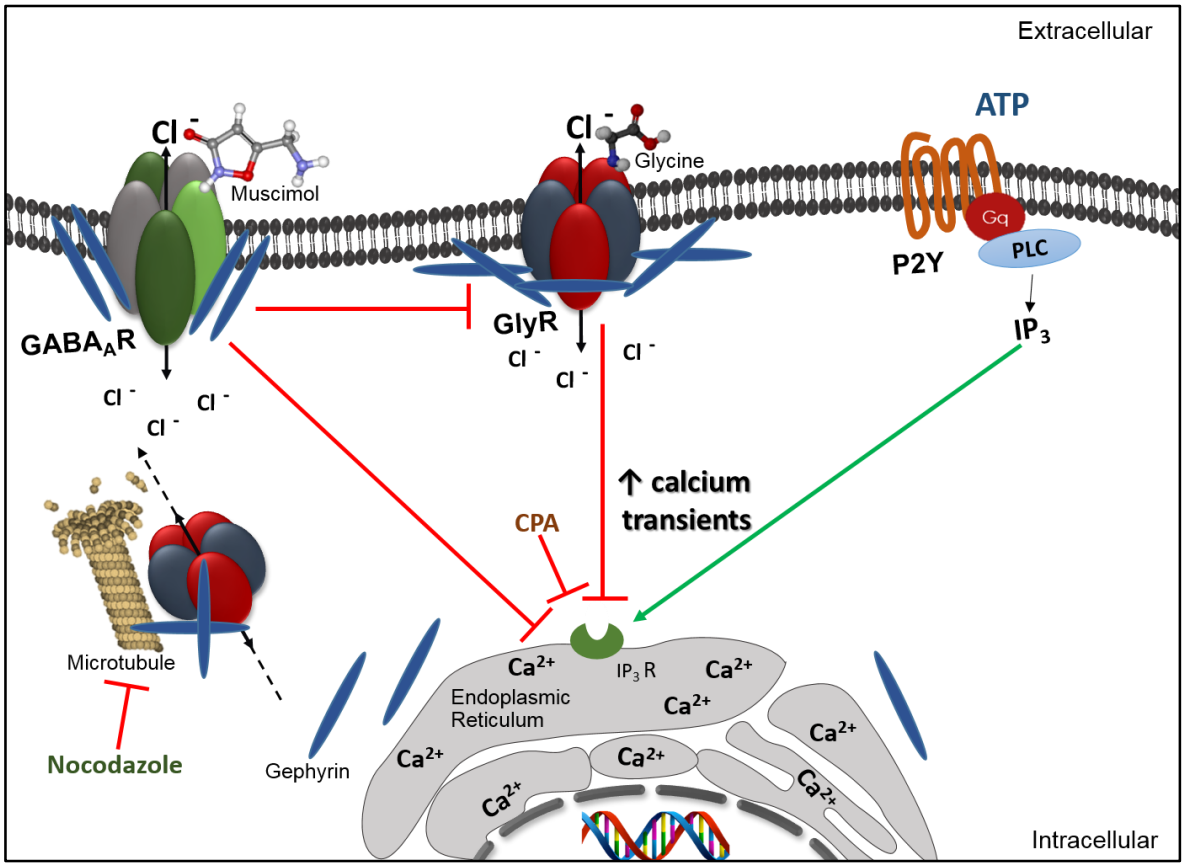


Figure 15: GlyR activation model in astrocytes. Glycine receptor activation, by glycine, inhibits calcium transients. The same effect is observed when $GABA_AR$ is activated by muscimol, which points to Cl^- as the effect mediator. The decrease in calcium transients is a result of decreased calcium release from the endoplasmic reticulum. As described in neurons, heteromeric GlyR recruitment to the astrocytes' cellular membrane, through gephyrin binding, is microtubule dependent.

6 | Conclusion and future perspectives

In conclusion, this work explores, for the first time, glycine receptor expression and function in brain astrocytes. Specifically, it shows that:

1) Glycine receptor is functionally expressed in astrocytes;
2) glycine receptor mediates an inhibitory effect, via chloride ion, in ATP-induced calcium transients, when activated by glycine;
3) $GABA_AR$ activation inhibits GlyR-mediated response;
4) glycine receptor activation inhibits calcium release from the endoplasmic reticulum.

Considering neurotransmission as a bidirectional path between astrocytes and neurons, the findings herein presented could have an impact in synaptic modulation and plasticity. In the future, and since astrocytes have the capability to contact hundreds of brain cells, it would be interesting to evaluate how astrocytic glycine receptor activation affects brain system network.

Furthermore, the modulation of astrocytic GlyR, and its consequence upon tonic glycinergic transmission, can be extremely interesting when considering potential strategies to treat brain disorders.

7 | References

1. Kandel E. R., Schwartz J. H. and Jessell T. M., eds (2013) Principles of Neural Science, 5th edn. McGraw-Hill, USA.
2. Zigmond, M. J., Bloom, F.E., Landis, S.C., Roberts, J.L. and Squire, L.R. (Editors) (1999) *Fundamental Neuroscience*. 1st Ed, Academic Press, U.S.A.
3. Dumoulin A., Rostaing P., Bedet C., Lévi S., Isambert M. F., Henry J. P., Triller A. and Gasnier B. (1999) Presence of the vesicular inhibitory amino acid transporter in GABAergic and glycinergic synaptic terminal boutons. J. Cell Sci. 112, 811–823.
4. Wang, D. D., & Bordey, A. (2008). *The Astrocyte Odyssey*. *Prog Neurobiol* (Vol. 86). doi:10.1016/j.pneurobio.2008.09.015.
5. Somjen, G. G. (1988). Nervenkitt: notes on the history of the concept of neuroglia. *Glia*, *1*(1), 2–9. doi:10.1002/glia.440010103
6. Allen, N. J., & Barres, B. a. (2009). Neuroscience: Glia - more than just brain glue. *Nature*, *457*(7230), 675–677. doi:10.1038/457675[a]
7. Araque, A., Parpura, V., Sanzgiri, R. P., & Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends in Neurosciences*, *22*(5), 208–215. doi:10.1016/S0166-2236(98)01349-6
8. Zhang, Y., & Barres, B. a. (2010). Astrocyte heterogeneity: an underappreciated topic in neurobiology. *Current Opinion in Neurobiology*, *20*(5), 588–94. doi:10.1016 /j.conb.2010.06.005
9. Lanciotti, A., Brignone, M., Bertini, E., Petrucci, T., Aloisi, F., & Ambrosini, E. (2013). Astrocytes: Emerging stars in leukodystrophy pathogenesis. Translational Neuroscience, 4(2), 144–164. doi:10.2478/s13380-013-0118-1
10. Barres, B. a. (2008). The Mystery and Magic of Glia: A Perspective on Their Roles in Health and Disease. *Neuron*, *60*(3), 430–440. doi:10.1016/j.neuron.2008.10.013
11. Sofroniew, M. V. and Vinters, H. V. (2010) Astrocytes: biology and pathology. *Acta Neuropathol,* 119, 7-35.
12. Oberheim, N. A., Goldman, S. A., & Nedergaard, M. (2012). Heterogeneity of Astrocytic Form and Function. *Methods Mol Biol*. 814: 23–45. doi:10.1007/978-1-61779-452-0_3.
13. Kimelberg, H. K. (2010). Functions of Mature Mammalian Astrocytes: A Current View. *The Neuroscientist*, *16*(1), 79–106. doi:10.1177/1073858409342593
14. Perea, G., Navarrete, M., & Araque, A. (2009). Tripartite synapses: astrocytes process and control synaptic information. *Trends in Neurosciences*, *32*(8), 421–431. doi:10.1016/j.tins.2009.05.001
15. Fellin, T., & Carmignoto, G. (2004). Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. *The Journal of Physiology*, *559*(1), 3–15. doi:10.1113/jphysiol.2004.063214
16. Perea, G., & Araque, A. (2010). GLIA modulates synaptic transmission. *Brain Research Reviews*, *63*(1-2), 93–102. doi:10.1016/j.brainresrev.2009.10.005
17. Allaman, I., Bélanger, M., & Magistretti, P. J. (2011). Astrocyte–neuron metabolic relationships: for better and for worse. *Trends in Neurosciences*, *34*(2), 76–87. doi:10.1016/j.tins.2010.12.001
18. Volterra, A., Liaudet, N., & Savtchouk, I. (2014). Astrocyte Ca(2+) signalling: an unexpected complexity. *Nature Reviews. Neuroscience*, *15*(5), 327–35. doi:10.1038/nrn3725
19. Perea, G., & Araque, A. (2005). Glial calcium signaling and neuron–glia communication. *Cell Calcium*, *38*(3-4), 375–382. doi:10.1016/j.ceca.2005.06.015
20. De Vuyst, E., Wang, N., Decrock, E., De Bock, M., Vinken, M., Van Moorhem, M., et al. (2009). Ca(2+) regulation of connexin 43 hemichannels in C6 glioma and glial cells. *Cell Calcium* 46, 176–187. doi: 10.1016/j.ceca.2009.07.002
21. Orellana, J. a, & Stehberg, J. (2014). Hemichannels: new roles in astroglial function. *Frontiers in Physiology*, *5*(June), 193. doi:10.3389/fphys.2014.00193
22. Siegel, R., Agranoff, B., Albers, R.W. and Molinoff, P. (Editors) (1989) *Basic Neurochemistry – Molecular, Cellular and Medical Aspects*. 4th Ed., Raven Press, U.S.A.
23. Johnson, J. W. and Ascher, P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature,* 325, 529-531.
24. Eulenburg, V. (2011). S.28.01 Glycine transporters: essential regulators of synaptic transmission. *European Neuropsychopharmacology*, *21*, S230. doi:10.1016/S0924-977X(11)70352-2

25. Eulenburg, V., Armsen, W., Betz, H., & Gomeza, J. (2005). Glycine transporters: essential regulators of neurotransmission. *Trends in Biochemical Sciences*, *30*(6), 325–333. doi:10.1016/j.tibs.2005.04.004
26. Xu, T.-L., & Gong, N. (2010). Glycine and glycine receptor signaling in hippocampal neurons: Diversity, function and regulation. *Progress in Neurobiology*, *91*(4), 349–361. doi:10.1016/j.pneurobio.2010.04.008
27. Danglot, L., Rostaing, P., Triller, A., & Bessis, A. (2004). Morphologically identified glycinergic synapses in the hippocampus. *Molecular and Cellular Neuroscience*, *27*(4), 394–403. doi:10.1016/j.mcn.2004.05.007
28. Aroeira, R. I., Sebastião, A. M., & Valente, C. a. (2014). GlyT1 and GlyT2 in brain astrocytes: expression, distribution and function. *Brain Structure and Function*, *219*(3), 817–830. doi:10.1007/s00429-013-0537-3
29. Aroeira, R. I., Ribeiro, J. a., Sebastião, A. M., & Valente, C. a. (2011). Age-related changes of glycine receptor at the rat hippocampus: from the embryo to the adult. *Journal of Neurochemistry*, *118*(3), 339–353. doi:10.1111/j.1471-4159.2011.07197.x
30. Aroeira R.I., Sebastião A.M. and Valente C.A. (2014) "BDNF, via truncated TrkB receptor, modulates GlyT1 and GlyT2 in astrocytes". GLIA, DOI: 10.1002/glia.22884
31. Vandenberg, R. J., Shaddick, K. and Ju, P. (2007) Molecular basis for substrate discrimination by glycine transporters. J Biol Chem, 282, 14447-14453.
32. Bowery, N. G., & Smart, T. G. (2006). GABA and glycine as neurotransmitters: a brief history. *British Journal of Pharmacology*, *147 Suppl* , S109–S119. doi:10.1038/sj.bjp.0706443
33. Dohi, T., Morita, K., Kitayama, T., Motoyama, N. and Morioka, N. (2009) Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. Pharmacol Ther, 123, 54-79.
34. Eulenburg, V., Armsen, W., Betz, H. and Gomeza, J. (2005) Glycine transporters: essential regulators of neurotransmission. *Trends Biochem Sci,* 30, 325-333.
35. Roux, M. J. and Supplisson, S. (2000) Neuronal and glial glycine transporters have different stoichiometries. *Neuron,* 25, 373-383.
36. Sato, K., Yoshida, S., Fujiwara, K., Tada, K. and Tohyama, M. (1991) Glycine cleavage system in astrocytes. *Brain Res,* 567, 64-70.
37. Gomeza, J., Hulsmann, S., Ohno, K., Eulenburg, V., Szoke, K., Richter, D. and Betz, H. (2003a) Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron, 40, 785-796.
38. Gomeza, J., Ohno, K., Hulsmann, S., Armsen, W., Eulenburg, V., Richter, D. W., Laube, B. and Betz, H. (2003b) Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron, 40, 797-806
39. Poyatos, I., Ponce, J., Aragon, C., Gimenez, C. and Zafra, F. (1997) The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. *Brain Res Mol Brain Res,* 49, 63-70.
40. Hanus, C. (2004). Intracellular Association of Glycine Receptor with Gephyrin Increases Its Plasma Membrane Accumulation Rate. *Journal of Neuroscience*, *24*(5), 1119–1128. doi:10.1523/JNEUROSCI.4380-03.2004
41. Langosch, D., Thomas, L. and Betz, H. (1988) Conserved quaternary structure of ligand-gated ion channels: the postsynaptic glycine receptor is a pentamer. *Proc Natl Acad Sci U S A,* 85, 7394-7398.
42. Dutertre, S., Becker, C.-M., & Betz, H. (2012). Inhibitory Glycine Receptors: An Update. *Journal of Biological Chemistry*, *287*(48), 40216–40223. doi:10.1074/jbc.R112.408229
43. Kirsch, J. (2006). Glycinergic transmission. *Cell and Tissue Research*, *326*(2), 535–540. doi:10.1007/s00441-006-0261-x
44. Lynch, J. W. (2009). Native glycine receptor subtypes and their physiological roles. *Neuropharmacology*, *56*(1), 303–309. doi:10.1016/j.neuropharm.2008.07.034
45. Grudzinska, J., Schemm, R., Haeger, S., Nicke, A., Schmalzing, G., Betz, H. and Laube, B. (2005) The beta subunit determines the ligand binding properties of synaptic glycine receptors. *Neuron,* 45, 727-739.
46. Avila, A., Nguyen, L., & Rigo, J.-M. (2013). Glycine receptors and brain development. *Frontiers in Cellular Neuroscience*, *7*(October), 1–11. doi:10.3389/fncel.2013.00184
47. Grenningloh, G., Pribilla, I., Prior, P., Multhaup, G., Beyreuther, K., Taleb, O. and Betz, H. (1990) Cloning and expression of the 58 kd beta subunit of the inhibitory glycine receptor. *Neuron,* 4, 963-970.

48. Levi, S. (2004). Gephyrin Is Critical for Glycine Receptor Clustering But Not for the Formation of Functional GABAergic Synapses in Hippocampal Neurons. *Journal of Neuroscience*, *24*(1), 207–217. doi:10.1523/JNEUROSCI.1661-03.2004
49. Tyagarajan, S. K., & Fritschy, J.-M. (2014). Gephyrin: a master regulator of neuronal function? *Nature Reviews Neuroscience*, *15*(3), 141–156. doi:10.1038/nrn3670
50. Charrier, C., Machado, P., Tweedie-Cullen, R. Y., Rutishauser, D., Mansuy, I. M., & Triller, A. (2010). A crosstalk between β1 and β3 integrins controls glycine receptor and gephyrin trafficking at synapses. *Nature Neuroscience*, *13*(11), 1388–1395. doi:10.1038/nn.2645
51. Kirsch, J. (2006). Glycinergic transmission. *Cell and Tissue Research*, *326*(2), 535–540. doi:10.1007/s00441-006-0261-x
52. Choii, G., & Ko, J. (2015). Gephyrin: a central GABAergic synapse organizer. *Experimental & Molecular Medicine*, *47*(4), e158. doi:10.1038/emm.2015.5
53. Tretter, V., Mukherjee, J., Maric, H.-M., Schindelin, H., Sieghart, W., & Moss, S. J. (2012). Gephyrin, the enigmatic organizer at GABAergic synapses. *Frontiers in Cellular Neuroscience*, *6*(May), 23. doi:10.3389/fncel.2012.00023
54. Kim, E. Y., Schrader, N., Smolinsky, B., Bedet, C., Vannier, C., Schwarz, G., & Schindelin, H. (2006). Deciphering the structural framework of glycine receptor anchoring by gephyrin. *The EMBO Journal*, *25*(6), 1385–1395. doi:10.1038/sj.emboj.7601029
55. Kirsch J., Langosch D., Prior P., Littauer U. Z., Schmitt B. and Betz H. (1991) The 93-kDa glycine receptor-associated protein binds to tubulin. J. Biol. Chem. 266, 22242–22245.
56. Meier J., Vannier C., Serge´ A., Triller A. and Choquet D. (2001) Fast and reversible trapping of surface glycine receptors by gephyrin. Nat. Neurosci. 4, 253–260.
57. Lynch, J. W. (2004). Molecular structure and function of the glycine receptor chloride channel. *Physiological Reviews*, *84*(4), 1051–1095. doi:10.1152/physrev.00042.2003
58. Pribilla, I., Takagi, T., Langosch, D., Bormann, J. and Betz, H. (1992) The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. *EMBO J,* 11, 4305-4311.
59. Gage, G. J., Kipke, D. R., Shain, W. Whole Animal Perfusion Fixation for Rodents. J. Vis. Exp. (65), e3564, doi:10.3791/3564 (2012).
60. Pfaffl, M. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), e45.
61. Jacob, P. F., Vaz, S. H., Ribeiro, J. a., & Sebastião, A. M. (2014). P2Y $_1$ receptor inhibits GABA transport through a calcium signalling-dependent mechanism in rat cortical astrocytes. *Glia*, *62*(8), 1211–1226. doi:10.1002/glia.22673
62. Lange, S. C., Bak, L. K., Waagepetersen, H. S., Schousboe, A. and Norenberg, M. D. (2012) Primary cultures of astrocytes: their value in understanding astrocytes in health and disease. *Neurochem Res,* 37, 2569-2588.
63. Belachew, S., Malgrange, B., Rigo, J. M., Rogister, B., Leprince, P., Hans, G., Moonen, G. (2000). Glycine triggers an intracellular calcium influx in oligodendrocyte progenitor cells which is mediated by the activation of both the ionotropic glycine receptor and Na+ - dependent transporters. *European Journal of Neuroscience*, *12*(6), 1924–1930.
64. Sigma-Aldrich. Nocodazole [Online]. Available: http://www.sigmaaldrich.com/catalog/product/sigma/m1404?lang=pt&region=PT&gclid=CjwKEAjwzJexBRCa_pGo8IK0ilASJABfGldbGpywCaXjqxhWSknw3zu91AFszMC5DpC1unqhy7__XBoC3NTw_wcB [Accessed 10 April 2015].
65. Sigma-Aldrich. Cyclopiazonic acid from Penicillium cyclopium [Online]. Available: http://www.sigmaaldrich.com/catalog/product/sigma/c1530?lang=pt&region=PT [Accessed 4 March 2015].
66. Harvey, R. J & Yee, B. K (2013). Glycine transporters as novel therapeutic targets in schizophrenia, alcohol dependence and pain. Nature Reviews Drug Discovery 12, 866–885 (2013) doi:10.1038/nrd3893.
67. Kaila, K., Price, T. J., Payne, J. a., Puskarjov, M., & Voipio, J. (2014). Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nature Reviews Neuroscience*, *15*(10), 637–654. doi:10.1038/nrn3819
68. Kirchner, A., Breustedt, J., Rosche, B., Heinemann, U. F. and Schmieden, V. (2003) Effects of taurine and glycine on epileptiform activity induced by removal of Mg2+ in combined rat entorhinal cortex-hippocampal slices. Epilepsia, 44, 1145-1152.
69. Aline Winkelmann et al. (2014). Changes in neural network homeostasis trigger neuropsychiatric symptoms. J Clin Invest; 124(2):696–711. doi:10.1172/JCI71472.

70. Li Y, Wu LJ, Legendre P, and Xu TL (2003a) Asymmetric cross-inhibition between GABAA and glycine receptors in rat spinal dorsal horn neurons. *J Biol Chem* 278:38637–38645.
71. Li Y and Xu TL (2002) State-dependent cross-inhibition between anionic GABAA and glycine ionotropic receptors in rat hippocampal CA1 neurons. Neuroreport 13:223–226.
72. Bekar, L. K., & Walz, W. (1999). Evidence for chloride ions as intracellular messenger substances in astrocytes. *Journal of Neurophysiology*, *82*(1), 248–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10400953

Appendix 1 | Tables

Table 1: List of primary antibodies

Antigen	Antibody	Host	Assay	Blocking	Dilution	Source
Glyceraldehyde-3-phosphate dehydrogenase	Anti-GAPDH	Mouse Monoclonal	WB		1:1000	ABCAM®
Glial fibrillary acidic protein	Anti -GFAP	Mouse Monoclonal	ICC/IHC	10% FBS in PBS	1:250	Sigma®
		Rabbit Polyclonal	ICC/IHC	10% FBS in PBS	1:500	Sigma®
Full Glycine Receptor	Anti-mAb4a	Mouse Monoclonal	WB		1:250	Synaptic Systems®
			ICC/IHC	0,25% gelatin in PBS	1:250	
Glycine Receptor α2 subunit	Anti-GlyRα2	Rabbit Polyclonal	ICC/ IHC	10% FBS in PBS	1:100	Santa Cruz®
Glycine Receptor β subunit	Anti β subunit	Goat Polyclonal	WB		1:200	Santa Cruz®
			ICC	10% FBS in PBS	1:50	
Gephyrin, a postsynaptic anchor of inhibitory receptors	Anti Gephyrin	Rabbit Polyclonal	WB		1:500	Synaptic Systems®
			ICC	10% FBS in PBS	1:100	

Table 2: List of secondary antibodies

Antibody	Assay	Dilution	Source
Goat anti-mouse HRP	WB	1:10000	Santa Cruz®
Goat anti-rabbit-HRP	WB	1:10000	Santa Cruz®
Goat anti-mouse Alexa 568	ICC/IHC	1:400	Invitrogen®
Goat anti-rabbit Alexa 488	ICC/IHC	1:400	Invitrogen®
Rabbit anti-goat Alexa 568	ICC/IHC	1:400	Invitrogen®

Table 3: qPCR primers

Gene	Type	Primer sequence (5' - 3')	Fragment	Species
β-actin	Fw	5'-AGCCATGTACGTAGCCATCC-3'	228 bp	Rattus norvegicus
	Rev	5'-CTCTCAGCTGTGGTGGTGAA-3'		
GlyRα1	Fw	5´-ACTCTGCGATTCTACCTTTGG-3´	300 bp	Rattus norvegicus
	Rev	5´-ATATTCATTGTAGGCGAGACGG-3´		
GlyRα2	Fw	5´-CAGAGTTCAGGTTCCAGGG-3´	330 bp	Rattus norvegicus
	Rev	5´-TCCACAAACTTCTTCTTGATAG-3´		
GlyRα3	Fw	5´-GTGAGACACTTTCGGACACTAC-3´	352 bp	Rattus norvegicus
	Rev	5´-GATGGGTCGAGGTCTAATGAATC-3'		
GlyRβ	Fw	5´-CTGTTCATATCAAGCACTTTGC-3´	223 bp	Rattus norvegicus
	Rev	5´-GGGATGACAGGCTTGGCAG-3´		

Table 4: List of drugs

Drug	Mediated Effect	Concentration	Source
ATP	P2YR agonist	10 µM	Invitrogen®
CPA (Cyclopiazonic acid)	Inhibitor of ER Ca^{2+} ATPases	10 µM	BIogen®
DMSO	Organic solvent	1%	Sigma®
Gabazine	$GABA_AR$ antagonist	10 µM	Abcam®
Glycine	GlyR agonist	10µM- 10mM	Abcam®
Muscimol	$GABA_AR$ agonist	3 µM	Sigma®
Nocodazole	Interferes with microtubules polymerization	1 µM for CI 10 µM for ICC	Sigma®
Strychnine	GlyR antagonist	0.8 µM	Sigma®

Appendix 2 | qPCR standard and melting curves

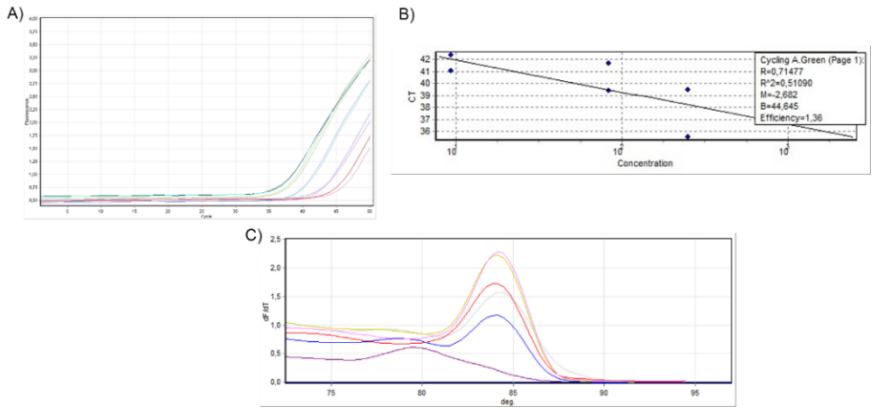


Figure 16: qPCR standard and melting curves analysis for GlyR α1. PCR amplification plot (A). Parameters calculated using standard curve created by plotting CP vs. the log concentration of cDNA (ng/µL) (B). Melting curve analysis (C).

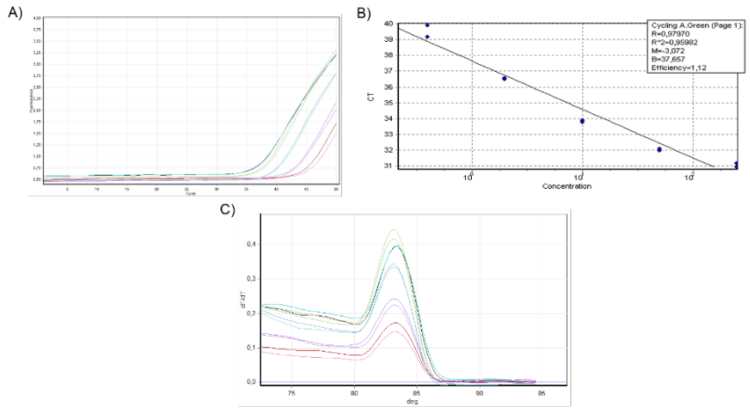


Figure 17: qPCR standard and melting curves analysis for GlyR α2. PCR amplification plot (A). Parameters calculated using standard curve created by plotting CP vs. the log concentration of cDNA (ng/µL) (B). Melting curve analysis (C).

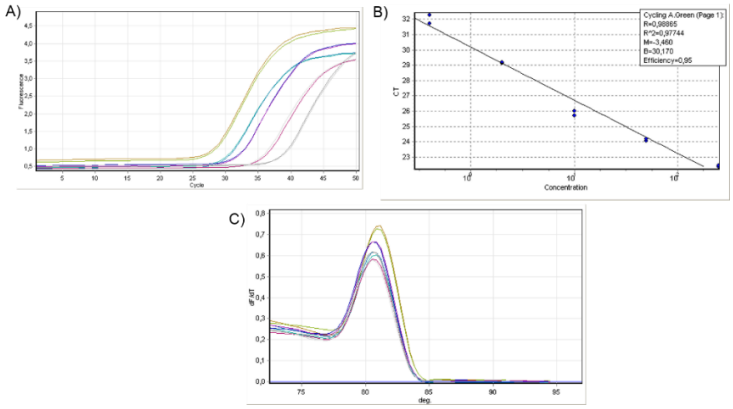


Figure 18: qPCR standard and melting curves analysis for GlyR β. PCR amplification plot (A). Parameters calculated using standard curve created by plotting CP vs. the log concentration of cDNA (ng/µL) (B). Melting curve analysis (C).

Appendix 3 | Fluorescence images

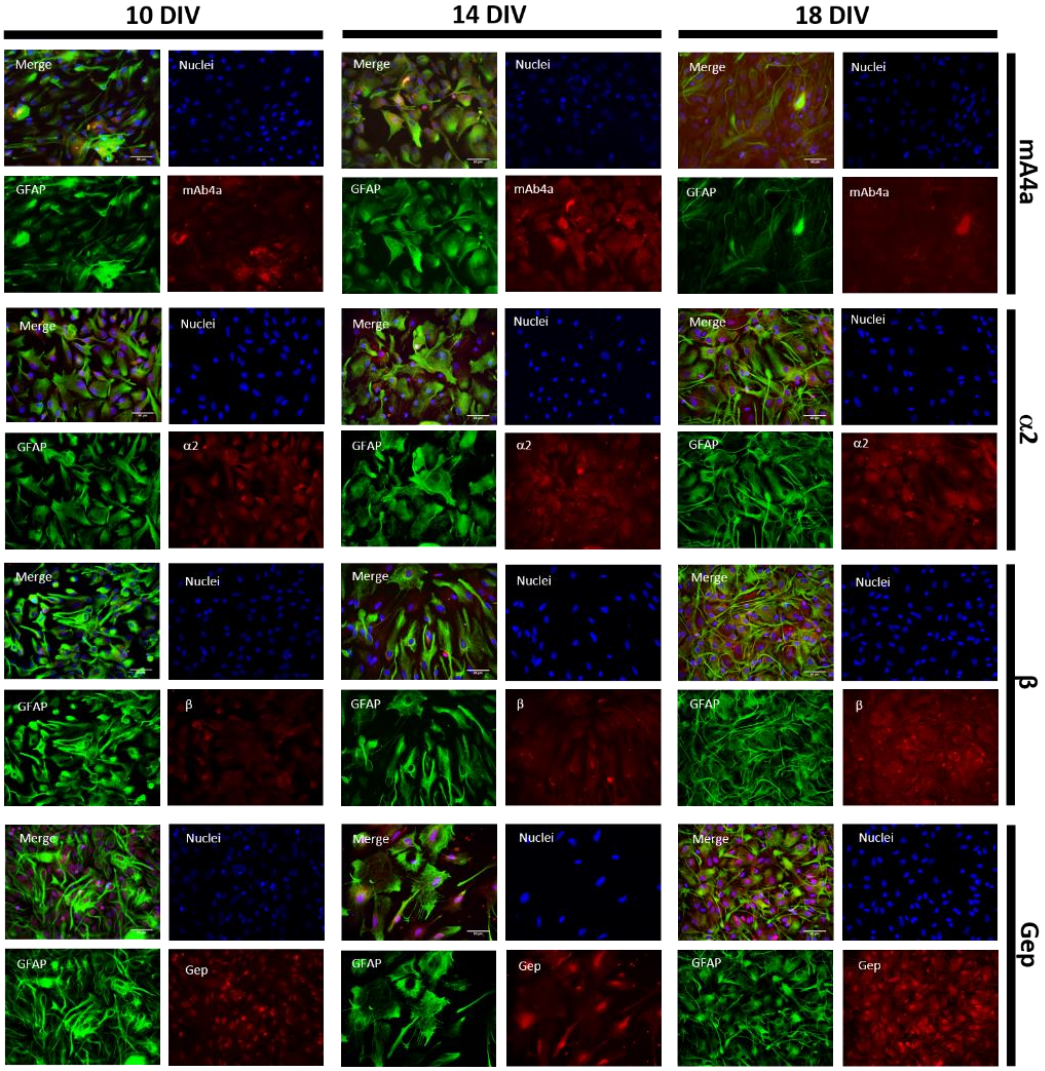


Figure 19: Double detection of mAb4a/α2/β/Gephyrin and GFAP in astrocytic cultures, at 10, 14 and 18 Days in vitro (DIV) per channel. Nuclei were stained with Hoechst, GFAP stained astrocytes are green and mAb4a/α2/β/Gephyrin immunoreactivity is red. Fluorescence images were acquired with a 40x objective in a Zeiss Axiovert 200. Dotted lines represent the amplified areas. Scale bar of 50 µm.

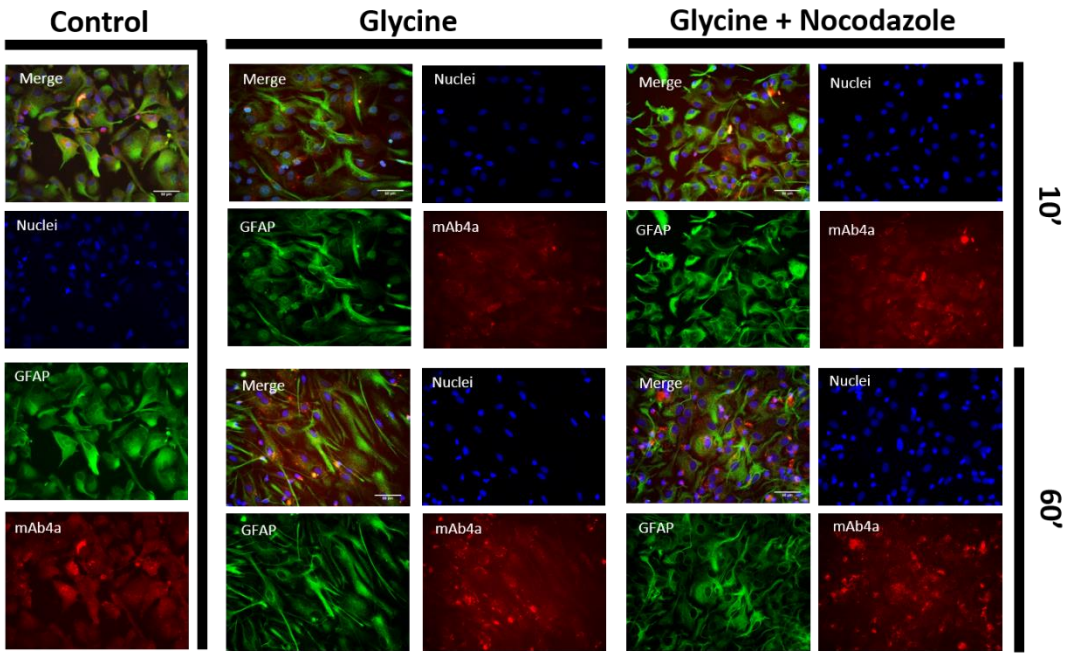


Figure 20: Double detection of GFAP and GlyR in 14 DIV astrocytes, in the presence of glycine and glycine + Nocodazole, for 10 or 60 min, per channel. Nuclei were stained with Hoechst, GFAP stained astrocytes are green and mAb4a immunoreactivity is red. Fluorescence images were acquired with a 40x objective in Zeiss Axiovert 200. Dotted lines represent the amplified areas. Scale bar of 50 µm.

Appendix 4 | Inhibitory dose - response curve

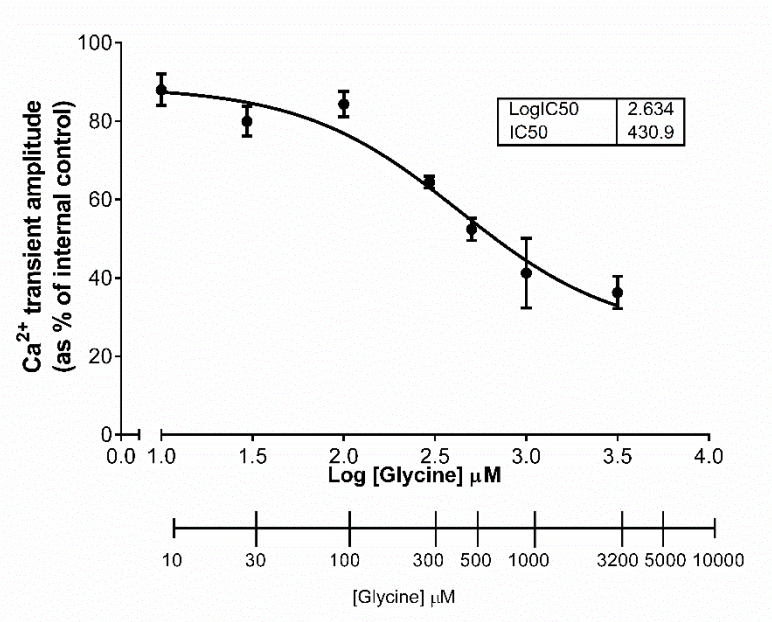


Figure 21: Inhibitory glycine dose-response curve. Each point of the curve represents the mean of the cellular response when cells are perfused with glycine in a dose range between 10-3200 µM. The adjustment curve was obtained by a non-linear regression of log(inhibitor) vs. response. All values are mean ± SEM. N=2-3 culture plates**.**

Appendix 5 | Calcium Imaging representative curves

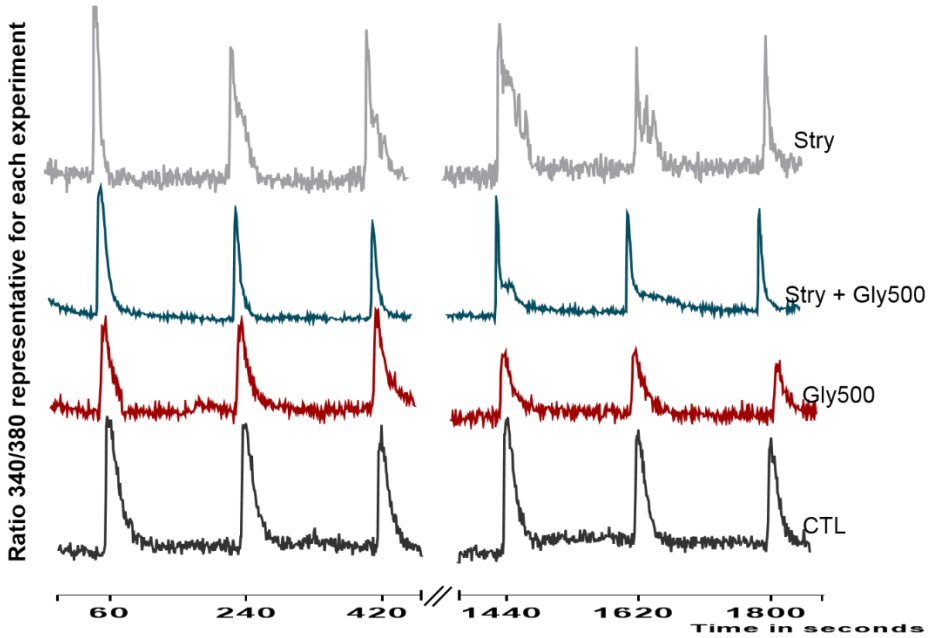


Figure 22: Representative curves of "GlyR activation upon ATP induced Ca^{2+} transients in cultured astrocytes" section. Each condition is indicated on the right side of the image. CTL, control; Gly500, glycine 500 μM; Stry + Gly500, Strychnine 0.8 μM + 500 μM; Stry, Strychnine 0.8 μM.

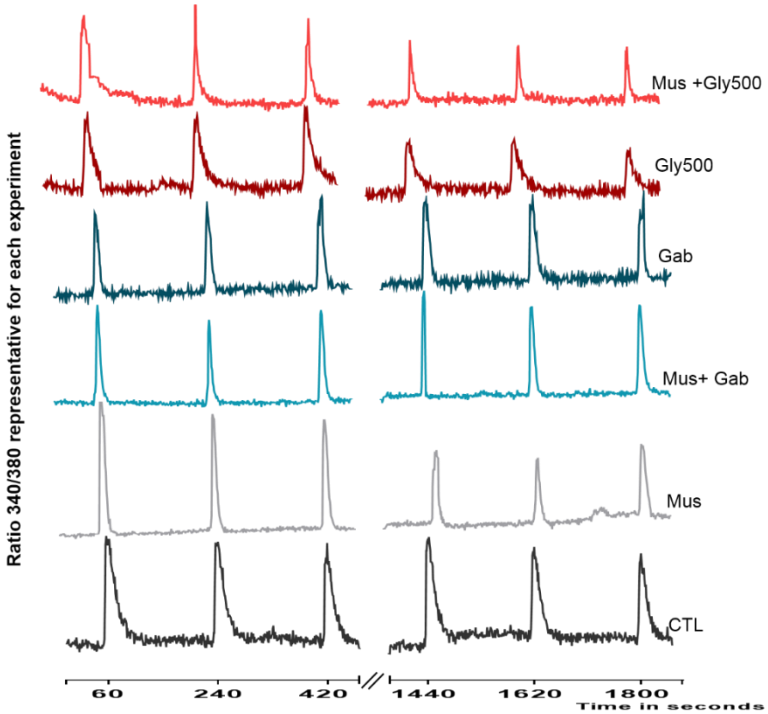


Figure 23: Representative curves of the "Cl^- mediates GlyR activation effect" section. Each condition is indicated on the right side of the image. CTL, control; Mus, muscimol 3 μM; Mus+Gab, muscimol 3 μM + gabazine 10 μM; Gab, gabazine 10 μM; Gly500, glycine 500 μM; Mus + Gly500, muscimol 3 μM + glicine 500 μM.

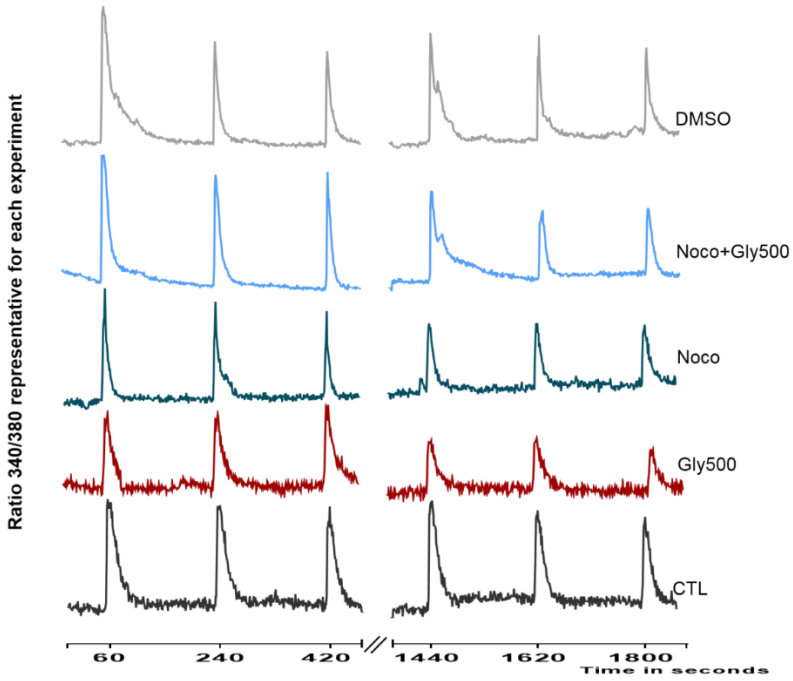


Figure 24: Representative curves of the "Nocodazole impairs GlyR activation effect upon ATP induced Ca^{2+} transients in cultured astrocytes" section. Each condition is indicated on the right side of the image. CTL, control; Gly500, glycine 500 µM; Noco, nocodazole 1 µM; Noco + Gly500, nocodazole 1 µM + glicine 500 µM; DMSO, DMSO 1%.

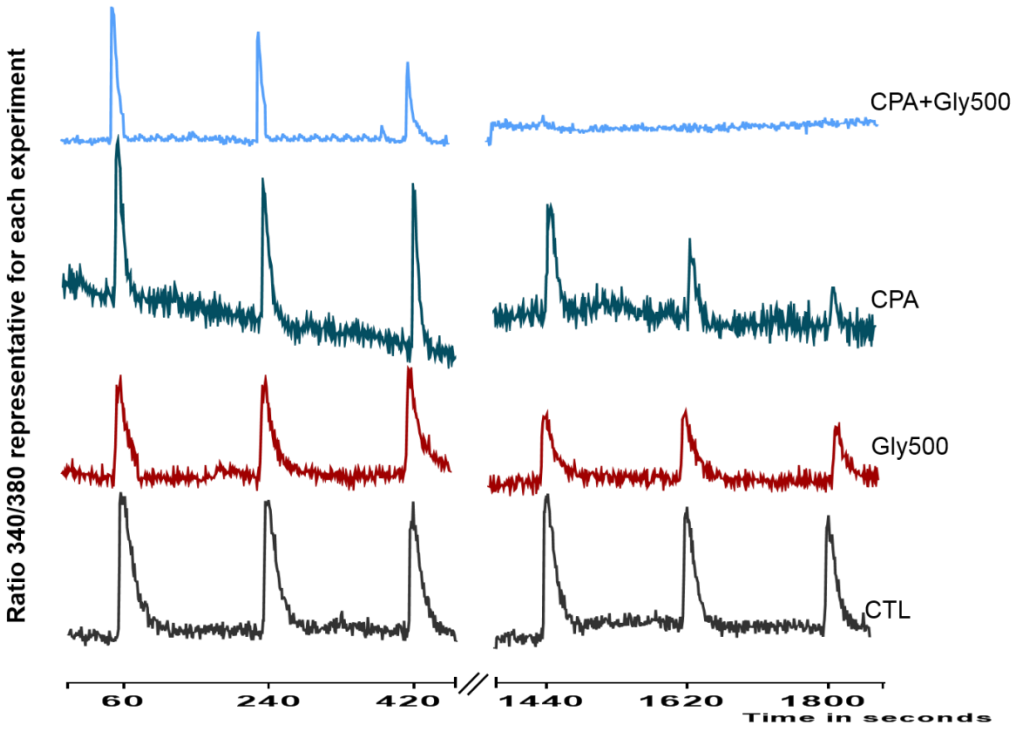


Figure 25: Representative curves of the "GlyR activation by glycine leads to a block of Ca^{2+} liberation from intracellular calcium stores in cultured astrocytes" section. CTL, control; Gly500, glycine 500 µM; CPA, CPA 10µM; CPA + Gly500, CPA 10 µM + glicine 500 µM.